

EFFECTS OF HIBERNATION ON TOOTH DEVELOPMENT ¹

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FOUR TEXT FIGURES AND ONE PLATE (SIX FIGURES)

INTRODUCTION

The marked depression of general metabolism during the hibernating state has been well known. Numerous studies have been made on the physiological and chemical aspects of the hibernating state and there have been a number of excellent reviews (Valentin, 1857; Dubois, 1899; Benedict and Lee, '38; Hook and Barron, '41), but apparently this is the first attempt to study the effect of hibernation on the developing tooth.

Since it has been shown previously that general metabolic changes are recorded accurately and chronologically in the teeth (Schour, '38; Sarnat and Schour, '41 and '42), we have undertaken to demonstrate quantitatively the effects of hibernation on tooth development. Two methods were employed: (1) intravital staining by means of alizarine red S to assess rate of dentin growth, and (2) notching of the teeth to assess rate of attrition.

¹ The authors are indebted to Dr. Isaac Schour and Dr. George F. Dick for their guidance in this study.

MATERIAL AND METHODS

The animals used in these experiments were thirteen-lined ground-squirrels (*Citellus tridecemlineatus* *Mitchilli*) caught in the vicinity of Chicago, Illinois, during the summer of 1940. These animals were selected because of their availability and ease with which hibernation can be induced. Nine controls and twenty-eight experimental animals of unknown age were used. The animals were kept in the laboratory for 2 months on standard rat ration. This consisted of 75% yellow corn-meal, 15% linseed-oil meal, 5% casein, 2% powdered alfalfa, 2% powdered yeast, 0.5% calcium carbonate and 0.5% sodium chloride. In addition, white baker's bread was supplied. The weight of the animals was determined to the nearest gram by means of a standard laboratory spring scale.

Hibernation was produced in the experimental group by withholding food and water for 2 days and then placing each animal in an individual cage in a dark, constant-temperature room kept at 2-5°C. The rate of attrition was studied by means of a transverse notch cut in the incisors with a jeweler's saw at the labio-gingival border. Its distance from the incisal edge was measured at intervals by calipers. These measurements calculated in millimeters per week furnished the rate of attrition which is essentially the same as the rate of eruption (Schour and Steadman, '35).

The rate of dentin apposition was determined by the alizarine red S method of vital staining (Schour, Hoffman, Sarnat and Engel, '41). Intraperitoneal injections of a 2% solution of alizarine red S (100 mg./kg.) were given. This dye was deposited in the dentin which was in process of forming and calcifying at the time of injection and appeared as a sharp red line in the ground section of the tooth.

The detailed procedure in these experiments conducted during the months of October, November and December 1940, and January 1941, was as follows:

Experimental group

21°C.	1st day —	Injected with alizarine red S
(4 days)	3rd day —	Weighed, placed in individually numbered cages without food or water
	5th day —	Weighed, transferred to constant-temperature room
2-5°C.	8th day —	Notched and measured incisors
(21 days)	8th to	
	26th day —	Observed and weighed every 2 to 5 days
	26th day —	Measured incisors, weighed, replaced to room temperature. Food and water <i>ad libitum</i>
21°C.	28th day —	Measured incisors, weighed, injected with alizarine red S
(4 days)	30th day —	Killed with ether, measured incisors and immediately fixed in 10% neutral formalin

*Control group*¹

	1st day —	Injected with alizarine red S
	3rd day —	Weighed
21°C.	5th day —	Weighed
(23 days)	10th day —	Notched and measured incisors
	22nd day —	Injected with alizarine red S
	24th day —	Killed with ether, measured incisors, weighed and immediately fixed in 10% neutral formalin

¹Food and water throughout total period.

Roentgenograms were taken of the femurs and the midsagittally sectioned heads after gross examination of the teeth. A standard dental x-ray machine was used (10 M.A., 65 K.V.). The distance from the tip of the cone to the dental occlusal film was 7 inches. The exposure was 7 seconds.

Ground and decalcified sections of the teeth were made after complete fixation of the heads. Longitudinal midsagittal ground sections (approximately 25-50 μ in thickness) were prepared of the upper left incisor by means of a moist revolving carborundum wheel. Transverse ground sections of the lower left incisors were prepared in a similar manner. The upper right incisor was washed, decalcified in 5% nitric acid, dehydrated, embedded in celloidin, sectioned serially in the longitudinal plane and stained with hematoxylin and

eosin. Ground sections were also made of the molars. All sections were mounted in damar.

The distance between the alizarine red S lines in the ground sections was measured by means of a filar micrometer eyepiece standardized to a stage micrometer, and the rate of dentin apposition calculated in micra per day. Comparable measurements were made of the hematoxylin and eosin sections. Any single measurement was determined by taking the average of three or more standardized readings. The usual site measured was at the level of the labial alveolar crest.

HISTOPHYSIOLOGY OF THE NORMAL GOPHER INCISOR

Gross examination reveals the fact that the incisor of the gopher closely resembles that of the rat. The upper and lower teeth are curved and tubular, possessing sharp incisal bevels which are more acute in the lowers. The upper incisor forms an arc of approximately 180 degrees, while the curvature of the lower is one of about 135 degrees (fig. 1) (Schour, '36). Both are segments of a spiral (Herzberg and Schour, '41).

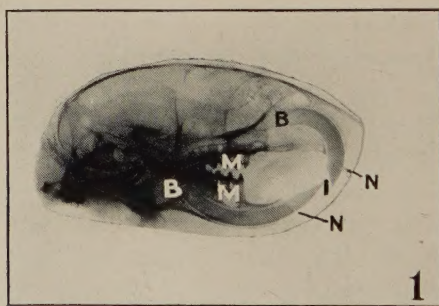


Fig. 1 Roentgenogram of left half of head of a control thirteen-lined ground-squirrel. N, notch made with jeweler's saw in upper and lower incisors to determine rate of eruption and attrition. I, incisal edge; B, basal end; M, molars.

The dentin, a very highly specialized calcified connective tissue, constitutes the bulk of the tooth and gives it form and strength. It increases in width from the basal to the incisal end (fig. 2). Dentin is continuously apposed at the pulpal border, one layer within the other. The organic matrix of

dentin (predentin) is laid down along the surface of the dental pulp. This is 5 to 20 μ in width and takes up the eosin color when stained with hematoxylin and eosin. Calcification of dentin proceeds normally in close chronological succession with its formation, through the deposition of globules that stain with hematoxylin. These globules increase in size and number and fuse until the dentin is homogeneously calcified.



Fig. 2 Photomicrograph of a midsagittal hematoxylin- and eosin-stained section of the upper right incisor and supporting structures of a control thirteen-lined ground-squirrel. La.Ging., labial gingiva; En.Sp., enamel space; Al.B., alveolar bone; P.D.M., periodontal membrane. $\times$ II. (Courtesy, I. Schour, *Anatomical Record*, vol. 65, p. 177, May, 1936.)

The tooth is supported on all surfaces except the labial by the periodontal membrane. On the labial side, adjacent to the papillary layer, it is supported by the periosteum of the alveolar bone. The alveolar bone forms the socket in which the incisor is supported.

A characteristic of all rodents is the continuous eruption and attrition of the incisors. These teeth move from the basal end outward. The upper and lower incisors are in occlusion and due to constant use the incisal edges wear

(attrition). Thus, as rapidly as the teeth move forward from the basal end, they are worn on the incisal edge. Consequently, the rate of eruption is essentially equal to the rate of attrition (Schour and Steadman, '35). So by making a transverse notch on the incisors, the amount the tooth moves outward (eruption) from the basal end can be determined by measuring the distance from the gingiva to the notch. In a similar manner the amount of tooth substance that is worn (attrition) may be determined by measuring from the notch to the incisal edge (fig. 3).

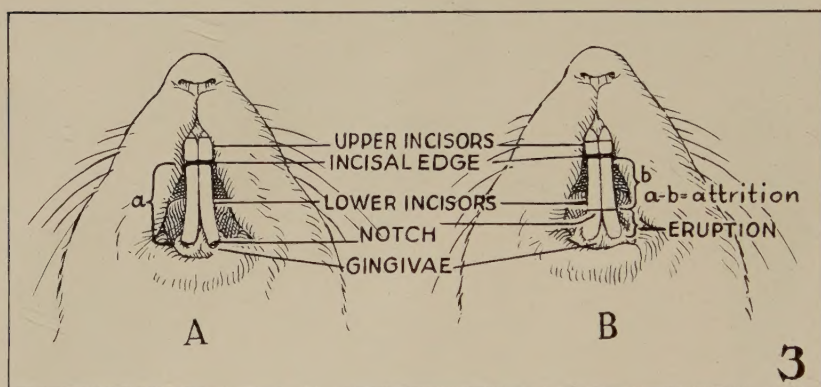


Fig. 3 Diagrams to show method of measuring the rates of eruption and attrition in the continuously growing incisors of the ground-squirrel. A, notch placed at gingival margin at beginning of experiment; B, location of the notch which has moved with the eruption of the tooth during the experiment. Amount of attrition is obtained by the difference between A and B. Amount of eruption is obtained by measuring distance notch has traveled from gingival margin.

In these experiments the rate of attrition was determined because, in contrast to eruption, there were two fixed points, the notch and the incisal edge, the distance between which was adequate for careful measurement. It was believed that accurate measurements of eruption during hibernation might not be obtained owing to dehydration with subsequent gingival recession; and because of marked suppression of eruption, the distance between the gingiva and notch would not be measurable.

TABLE 1

*Rate of weight change (grams per day) of thirteen-lined ground-squirrels
2-5°C. and 21°C.*

GROUP	NO.	SEX	ORIGINAL WEIGHT	TIME AT 2-5°C.	HIBER- NATION	21°C.	2-5°C.	21°C.
			<i>gm.</i>	<i>days</i>	<i>% of time</i>	<i>gm. lost</i> ¹	<i>gm. lost</i> ²	<i>gm. gained</i> ³
I	7	♂	184	21	100	10.0	1.3	5.5
	16	♀	176	21	100	9.5	0.9	5.0
	50	♂	307	59	95	12.5	1.5	5.0
	58	♀	260	59	90	17.0	1.3	5.5
	2 *	♀	198	21	90	7.0	2.2	6.0
	49	♂	196	59	88	5.0	1.5	3.0
	29	♂	142	21	86	9.5	1.3	5.0
	25	♂	174	21	86	6.5	1.8	3.0
					Average	10.0	1.4	4.8
II	6	♂	184	21	71	7.5	2.4	4.5
	24	♂	174	21	71	9.5	2.7	10.0
	3	♂	177	21	52	6.0	2.5	6.5
	9	♂	189	21	52	9.0	2.9	5.0
	13	♀	208	21	43	11.5	3.4	6.0
	14	♀	200	21	43	10.0	2.4	8.0
	1 d		201	21	24	10.5	2.3	...
					Average	9.1	2.7	6.7
III	19	♀	175	15	20	7.0	5.1	11.0
	8	♀	169	15	7	9.5	4.9	9.0
	26 d	♀	164	13	0	7.5	4.5	...
	4 d	♀	235	15	0	10.0	6.6	...
	5 d	♀	205	15	0	8.0	7.3	...
	10 d	♀	218	13	0	9.0	7.9	...
	12 d	♂	238	15	0	8.5	7.7	...
	20 d	♂	175	13	0	10.0	2.6	...
	30 d	♂	...	15	0	...	5.2	...
	23 d	♂	172	15	0	9.5	5.9	...
	15 d	♀	169	13	0	8.0	6.7	...
	18 d		155	10	0	6.5	5.4	...
	21 d		180	10	0	8.0	5.3	...
					Average	8.5	5.8	...

¹ No food, no water first and second days.

² No food, no water.

³ Standard diet — 2 days.

d Died before second injection of alizarine red S.

* No. 2 not included in summary table.

OBSERVATIONS

Weight. There was no significant weight change in the control group kept at room temperature on standard ration.² In the experimental group, however, there was significant weight change (table 1). The greatest weight loss occurred during the first 2 days at 21°C., after withdrawal of food and water; while at 2-5°C. the loss in weight was not as great and was related to the per cent of time the animals hibernated. When the animals were replaced at laboratory conditions and given food and water there was a marked increase in weight.

TABLE 2
Rate of incisal attrition (millimeters per week) of thirteen-lined ground-squirrel at 21°C.

NO.	SEX	WEIGHT	TIME BETWEEN MEASUREMENTS	UPPER INCISORS	LOWER INCISORS
		<i>gm.</i>	<i>days</i>	<i>mm. per week</i>	
32	♂	137	14	0.6	1.8
33	♀	143	14	1.4	1.0
36	♀	149	14	0.6	0.9
37	♀	138	14	0.8	1.2
38	♀	130	14	0.7	1.1
39	♀	161	14	0.7	0.5
40	♀	147	14	0.8	1.1
41		125	14	0.5	1.1
44	♂	193	14	1.3	1.5
				Average	0.8 1.1

Gross and roentgenographic studies of the skulls, incisors, molars and femurs revealed no significant differences between hibernating and non-hibernating animals.

Attrition. The rate of attrition in the control animals varied from 0.6 to 1.4 (average 0.8) and 0.5 to 1.8 (average 1.1) mm. per week for the upper and lower incisors respectively (table 2).

In the hibernating animals kept at 2-5°C. (table 3) the rate of incisal attrition was markedly suppressed — approximately in proportion to the per cent of time the animals hibernated. The experimental animals were grouped as follows:

² Injection of alizarine red S causes temporary loss of weight.

TABLE 3

Rate of incisal attrition (millimeters per week) of thirteen-lined ground-squirrel at 2-5°C. and 21°C.

GROUP	NO.	TIME BETWEEN MEASURE- MENTS	HIBERNATION	2-5°C.		21°C.		21°C.	
				Upper incisors	Lower incisors	Upper incisors	Lower incisors	Upper incisors	Lower incisors
I	7	days	% of time ¹	mm. per week		mm. per week ²		mm. per week ³	
	16	16	100	0.0	0.0	2.4	1.9	0.3	0.7
	16	16	100	0.1	0.0	2.6	2.3	0.3	0.4
	50	14	100	0.0	0.0	1.8	3.5	1.1	1.0
	58	14	100	0.0	0.0	0.9	1.4	0.7	0.6
	24	14	100	0.1	0.1	2.0	1.4	0.3	0.7
	6	16	94	0.2	0.4	0.2	0.3	0.1	0.1
	29	16	81	0.1	0.0	1.3	1.4	0.1	0.3
	25	16	81	0.0	0.2	1.1	2.1	1.3	0.1
Average				0.1	0.1	1.5	1.8	0.5	0.5
II	13	14	71	0.3	1.6				
	14	14	71	0.4	1.2				
	1	16	63	0.1	0.6				
	9	14	50	0.1	0.5				
	Average			0.2	1.0				
III	19	10	20	0.5	3.3				
	5	8	0	0.2	1.0				
	10	6	0	0.1	0.2				
	26	6	0	0.1	0.4				
	30	8	0	0.5	3.3				
	23	8	0	0.7	2.6				
	15	6	0	1.0	4.5				
Average				0.4	2.2				

¹ This represents the approximate period the animals hibernated when measurements for attrition were made.

² First and second day after hibernation.

³ Third and fourth day after hibernation.

Group I consisted of eight animals which hibernated from 81-100% of the period when measurements were taken. The average rate of attrition was 0.1 mm. per week for both the upper and lower incisors (upper incisors 87.5% suppression, lower incisors 91% suppression). These averages, however, do not reflect the exact effect of hibernation on the rate of attrition because in three of the animals which hibernated

throughout this experimental period (nos. 7, 50 and 58) no change was seen in the gingival-notch relationship at any time. This is also evidence of no eruption. In animals nos. 16, 24 and 29, there was 0.1 mm. or less attrition per week. In animals nos. 6 and 25 (which hibernated 94% and 81% of the time respectively), however, the rates of attrition were 0.2 (upper) and 0.4 (lower), and 0.0 (upper) and 0.2 (lower) mm. per week.

Group II consisted of four animals which hibernated from 50 to 71% of the time. The average rates of attrition were 0.2 and 1.0 mm. per week for the upper and lower incisors respectively. These rates varied for the uppers from 0.1 to 0.4 and for the lowers from 0.5 to 1.6 mm. per week (table 3).

Group III consisted of seven animals which hibernated from 0 to 20% of the time. The average rates of incisal attrition were 0.4 for the uppers and 2.2 mm. per week for the lowers. The lower incisors of animals nos. 15, 23 and 30, which did not hibernate, showed rates of attrition higher than the normal (table 3).

During the first 2 days after hibernation, the average rate of attrition of the eight animals which hibernated 81–100% of the time was 1.5 and 1.8 mm. per week for the upper and lower incisors. The average rate during the third and fourth day post-hibernation was 0.5 mm. per week for both the upper and lower incisors (table 3 and fig. 4a).

Dentin apposition. In the ground sections of the teeth for each intraperitoneal injection of alizarine red S a distinct red line was found in the incisor dentin formed and calcified at the particular time. In the molar dentin, however, no alizarine red S was seen. Traces of alizarine red S were found in the surrounding alveolar bone. By means of the hand lens, the alizarine red S lines were seen to be much closer in the teeth of the animals which hibernated than in the controls.

The average rate of dentin apposition in the nine control animals was $12.8\ \mu$ per day. The distance between the alizarine red S lines for the control period varied from 211 to $336\ \mu$ (fig. 5). Hence the daily rate of dentin apposition varied from

10.0 to 16.0 μ (table 4). This was in marked contrast to the rate of dentin apposition of the animals kept at 2–5°C. The average rate of dentin apposition per day for the period when the animals were kept at 2–5° was calculated by subtracting 76.8 μ from the distance between the alizarine red S lines. This was done to correct for the 4 days before and 2 days after when the animals were at room temperature and the alizarine red S was administered. $6 \times 12.8 \mu$ (average rate of dentin apposition per day in control animals) = 76.8 μ .

TABLE 4

Rate of dentin apposition of thirteen-lined ground-squirrel incisors at 21°C.

NO.	TIME BETWEEN ALIZARINE RED S INJECTIONS	DISTANCE BETWEEN ALIZARINE RED S LINES	RATE OF DENTIN APPPOSITION PER DAY
	<i>days</i>	μ	μ
32	21	319	15.2
33	21	273	13.0
36	21	336	16.0
37	21	211	10.0
38	21	229	10.9
39 ¹	23	261	11.3
40	21	298	14.2
41	21	285	13.6
44	21	232	11.0
			Average 12.8

¹ No second injection of alizarine red S. Measured to pulpal border.

Group I consisted of six animals which hibernated from 86–100% of the time; the average rate of dentin apposition was 1.5 μ per day or 12% of the control (table 5). This was probably higher than the actual rate because the period was also included when the animals were first placed in the cold and did not hibernate. Three of these animals (nos. 49, 50 and 58) were kept at 2–5°C. for 59 days, while the remaining three animals of this group (nos. 7, 16 and 29) were kept at 2–5°C. for 21 days (figs. 6 and 10). Animal no. 6 was not included in this group. Although this animal hibernated 94% of the time, the rate of dentin apposition was 4.3 μ per day. This animal also had a higher rate of attrition than the other animals of this group (table 3).

TABLE 5

Rate of dentin apposition of thirteen-lined ground-squirrel incisors at 2-5°C.

GROUP	NO.	TIME BETWEEN ALIZARINE RED S INJECTIONS	DISTANCE BETWEEN ALIZARINE RED S LINES	RATE OF DENTIN APPOSITION PER DAY	HIBERNATION
		<i>days</i>	μ	μ	<i>% of time</i>
I	7	27	102	1.2	100
	16	27	106	1.4	100
	50	65	145	1.2	95
	58	65	163	1.5	90
	49	65	189	1.9	88
	29	27	109	1.5	86
				Average 1.5	
II	24	27	125	2.3	71
	3	27	108	1.5	52
	9	27	149	3.4	52
	13	27	126	2.3	43
	14	27	103	1.3	43
	1 d	27	165	4.2	24
				Average 2.5	
III	19	21	115	2.5	20
	8	21	200	8.2	7
	26 d	17	79	2.1	0
	4 d	19	174	8.2	0
	5 d	19	159	7.2	0
	10 d	17	152	7.7	0
	12 d	19	155	6.9	0
	20 d	17	130	6.0	0
	30 d	19	136	5.7	0
	23 d	19	166	7.6	0
	15 d	17	157	8.1	0
	18 d	17	134	8.3	0
	21 d	14	126	7.5	0
				Average 6.6	

d Died before second injection of alizarine red S.

The average rate of dentin apposition per day for the period when the animals were kept at 2-5°C. was calculated by subtracting 76.8 μ from the distance between the alizarine red S lines. This was done to correct for the 4 days before and 2 days after when the animals were at room temperature and the alizarine red S was administered.

$6 \times 12.8 \mu$ (average rate of dentin apposition per day in control animals) = 76.8 μ .

Group II consisted of six animals which hibernated from 24 to 71% of the time; the daily rate of dentin apposition varied from $1.3\ \mu$ to $4.2\ \mu$. The average rate was $2.5\ \mu$ or 20% of the control (table 5).

Group III consisted of thirteen animals which hibernated between 0 and 20% of the time; the average rate was $6.6\ \mu$ per day, or 52% of the control (table 5). In this group, eleven of the thirteen animals died, hence no second injection of alizarine red S could be given and the measurements were made from the alizarine red S line to the pulpal border. With the exception of animals nos. 19 and 26, the rate of dentin apposition varied from 5.7 to $8.3\ \mu$ per day (fig. 4b).

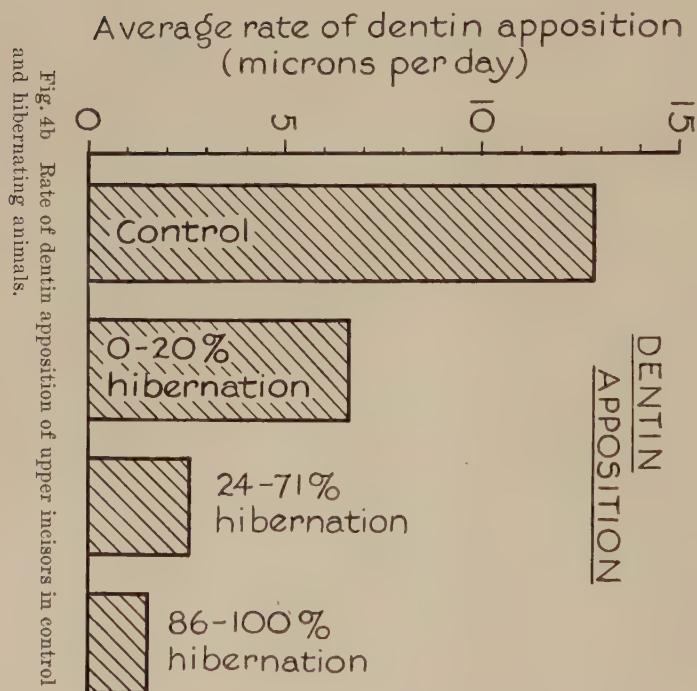
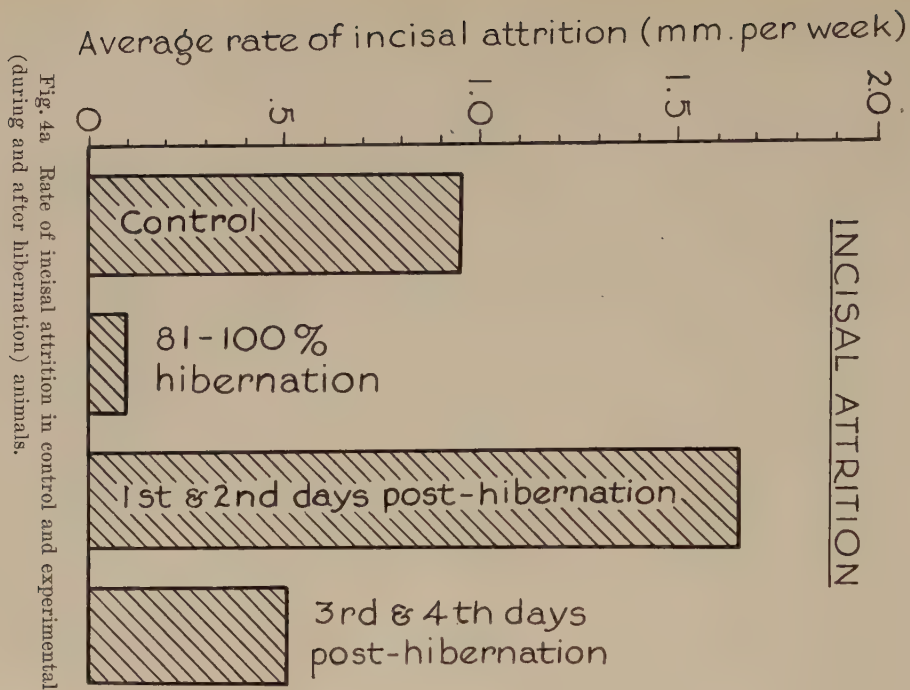
Hematoxylin and eosin sections of the incisors showed in the dentin of both the control and experimental animals the limits of zones (figs. 7, 8) which by micrometer measurements were found to correspond closely with the time of injection of alizarine red S. The beginning of the zone was sharply demarcated by a narrow light-staining stripe immediately followed by a narrower deep hematoxylin-stained stripe (calciotraumatic line). Comparable but less distinct findings were seen at the end of the zone at the pulpal border (figs. 9, 10).

The dentin between the first and second calciotraumatic lines was well formed and calcified in both the experimental and the control animals, but in the former group the incremental pattern was not based on a daily rhythm. No significant changes were found in the pulp, odontoblasts, enamel organ or alveolar bone.

*Statistical evaluation*³

Correlation coefficients and their standard deviations were computed separately for weight changes, rates of incisal attrition and dentin apposition during hibernation by the standard Pearson equation (table 6). By each criterion the correlation is most probably significant. In every case the

³ By Mr. Robert R. Williamson, Department of Mathematical Biophysics, University of Chicago.



correlation coefficient is greater than 2.3 times the standard deviation of the correlation coefficient. It can be said with a high degree of statistical certainty that hibernation produces a definite inhibition of all the variables (fig. 4c).

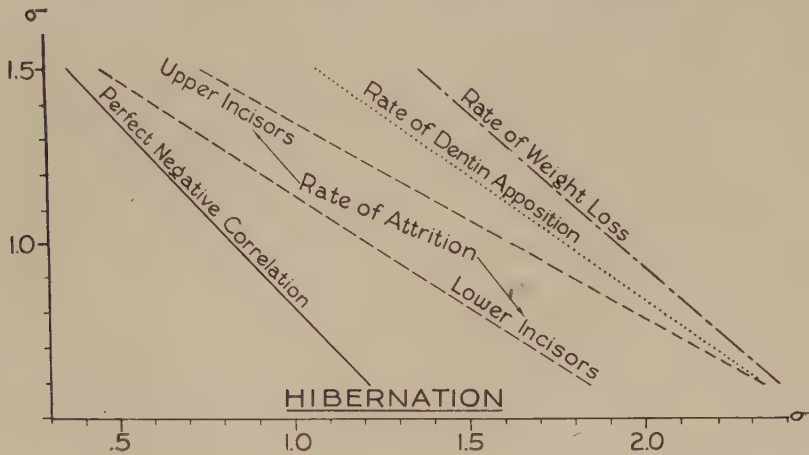


Fig. 4c Rates of weight loss, incisal attrition and dentin apposition plotted against hibernation time with both axes in units of standard deviation. The lines are the best fitting obtained by the least square method. Note the close parallel to perfect negative correlation.

It is probable that if time of hibernation were determined more accurately, and the animals were not disturbed for weighing, the correlations would have been better. Rates of dentin apposition and incisal attrition could very well be used as a measure of hibernation in future experiments. These

TABLE 6

Statistical evaluation of rates of weight loss, incisal attrition and dentin apposition during hibernation.

VARIABLE	CORRELATION COEFFICIENT (σ)	STANDARD DEVIATION OF CORRELATION COEFFICIENT (σ_r)	(σ/σ_r)
Weight loss	.862	.192	4.49
Incisal attrition			
Upper	.549	.229	2.40
Lower	.622	.229	2.72
Dentin apposition	.672	.200	3.36

methods have the advantage of offering a physical measure, readily determined with a high degree of accuracy without disturbing the animal during hibernation. In addition, the rate of dentin apposition is permanently recorded in the ground section of the tooth by the alizarine red S.

DISCUSSION

Attrition and eruption. The result of these experiments demonstrated that hibernation caused a "physiological arrest" of eruption and attrition in the incisors of the thirteen-lined ground-squirrel. These were additional manifestations of the marked metabolic depression which occurred when the animals passed from the non-hibernating to the hibernating state.

In the post-hibernation period the marked increase in incisal wear (and eruption) during the first 2 days (fig. 4a) may be attributed to the ingestion of food and sudden increase in metabolism.

Relatively few studies have been made on the metabolic factors which affect rate of eruption. Massler and Schour ('41) reviewed the subject of tooth eruption. Schour and van Dyke ('32) demonstrated that hypophysectomy retarded the rate of eruption of the incisors in the white rat. Herzberg and Schour ('41) showed that the administration of thyroxine to white rats increased the rate of eruption.

The rate of eruption was retarded on a local basis by placing metal crowns on the incisal edges (Massler and Schour, '41). The crowns did not wear, the teeth remained in occlusion, and consequently eruption did not occur. In contrast, we observed increased rates of eruption where the opposing incisor was fractured or absent. These animals were not included in this study.

Dentin and bone. The rate of dentin apposition was markedly altered in deficiencies of vitamins A (Schour, Hoffman and Smith, '41) and C (Boyle, Bessey and Howe, '40) and magnesium (Gagnon and Patras, unpublished). The marked suppression of the activity of the dentin-forming cells of

animals kept at cold temperatures and hibernating for different periods of time was the only instance in which the rate of dentin apposition and calcification was altered without morphologic changes. Because hibernation decreased the rate only, with no disproportion in dentin formation, no "hibernation line" was seen.

Long-bone growth during hibernation also has been shown to be retarded (Cassens, '39). In the roentgenograms of the femurs studied in the control and experimental animals of our group no lines of increased density were seen either for the period of induced hibernation or hibernation that may have occurred during previous winters before the animals were obtained. Harris ('33) has described "lines of arrested growth" in roentgenograms of long bones. These lines of increased density were attributed to faulty metabolism during periods of illness. It has been shown, moreover, that lines of increased density on the roentgenograms may be caused by a lack of resorption of trabecular bone (Adams and Sarnat, '40). During hibernation, however, all of the processes of growth, calcification and resorption, both cartilaginous and osseous, are retarded. Consequently there is no "line of arrested growth."

No significant differences were seen in the calcification of the dentin in the experimental and control animals. The calcification was normal for the period demarcated by the alizarine red S injections although in some animals the pre-experimental dentin was not well calcified. In the normal animal a hypocalcified layer followed by a narrow hypercalcified layer is laid down every 24 hours (Schour, Chandler and Tweedy, '37). The constancy of the incremental pattern in the dentin is not maintained during hibernation. In the hibernating animals the incremental pattern occurs over a longer period of time.

Studies of blood calcium and phosphorus during hibernation have been controversial. Various calcium values have been reported (Cassens, '39; Suomalainen, '38). No change was found by Cassens ('39) in the inorganic phosphorus of the

blood of hibernating hedgehogs. Suomalainen ('38) found in hedgehog serum that during hibernation the magnesium increased. He also reported that the injection of magnesium chloride and insulin into hedgehogs will produce a state closely resembling hibernation.

Schour (Schour, Chandler and Tweedy, '37) described the "calciotraumatic line," a non-specific reaction of dentin calcifying at the particular time of a change in the animal's metabolism. This line, a narrow, eosin-staining stripe followed by a narrower hematoxylin-stained stripe, has been seen in animals subjected to parathyroidectomy (Schour, Chandler and Tweedy, '37), hypophysectomy (Schour and van Dyke, '32), parathormone injections (Schour, Tweedy and McJunkin, '34), and diets containing yellow phosphorus (Adams and Sarnat, '40) or fluorine (Schour and Smith, '34).

In many of the decalcified hematoxylin- and eosin-stained sections of the upper incisors of our animals a distinct zone separated at the beginning and end by calciotraumatic lines could be seen. The findings were similar in the control and experimental animals, and the lines corresponded closely to the time of injection of alizarine red S. Thus an injection of alizarine red S may cause a calciotraumatic line in the dentin matrix (figs. 7, 8, 9 and 10).

In the ground sections of the molars no alizarine red S was seen in the dentin. Inasmuch as the molars, in contrast to the incisors, were teeth of limited growth, dentin apposition had been completed at the time of injection of alizarine red S. In the surrounding alveolar bone which was being continuously apposed and resorbed, however, alizarine red S was seen.

Weight. Changes in body weight during hibernation have been studied (Valentin, 1857; Dubois, 1899; Benedict and Lee, '38; and Hook and Barron, '41). In general, the loss of body weight in the period of starvation during the hibernating state was markedly less than during the period of starvation in the non-hibernating state (warm and cold temperatures). In the literature early reports showed that some animals actually gained in weight. It has been demonstrated,

however, that this was not a true physiologic gain in weight but was most probably due to the hygroscopic affinity of fur (Benedict and Lee, '38). In our experiments the changes in body weight were studied to correlate approximately general metabolic changes with dental changes.

As was expected during the initial 2 days without food or water (at room temperature), the weight loss varied from 5 to 17 gm. per day (table 1). In the animals kept at 2-5°C. the daily weight loss was greatest in those which hibernated least. Conversely, those animals which hibernated most had the smallest weight loss (table 1). These findings are consistent with those of the rate of attrition and dentin apposition. When the animals were returned to standard laboratory conditions and given food and water the average daily gain in weight for the 2 days after hibernation varied from 3 to 11 gm. There was a slight transient weight loss in some of the animals at the beginning and end of the experimental and control periods following the injection of alizarine red S.

SUMMARY AND CONCLUSIONS

The effects of hibernation on tooth development in the thirteen-lined ground-squirrel was studied in twenty-eight animals. Nine animals were used as controls. The animals were subjected to (1) notching of the incisors to determine the rates of attrition and eruption and (2) intravital staining with alizarine red S to determine the rates of dentin apposition. The changes in body weight were observed during these experiments.

The essential findings were:

1. No changes were found in the roentgenograms and gross examination of the skulls, incisors, molars and femurs of animals subjected to hibernation.

2. The weight loss was inversely proportional to the time the animal hibernated. The average weight loss was: Group I (86-100% hibernation), 1.4 gm. per day; group II (24-71% hibernation), 2.7 gm. per day; and group III (0-20% hibernation), 5.8 gm. per day (table 7).

TABLE 7

Summary.

Average rate of attrition and dentin apposition of thirteen-lined ground-squirrel incisors and weight loss at 2-5°C.

GROUP	HIBERNATION	RATE OF ATTRITION		RATE OF DENTIN APPPOSITION	WEIGHT LOSS	
		Upper	Lower		21°C. ¹	2-5°C. ²
	% of time ³	mm. per week		μ per day	gm. per day	
I	81-100	0.1	0.1	1.5	10.0	1.4
II	24- 71	0.2	1.0	2.5	9.1	2.7
III	0- 20	0.4	2.2	6.6	8.5	5.8

¹ No food, no water, for 2 days.

² No food, no water.

³ Approximate period during which animals were found in the state of hibernation.

TABLE 8

Summary.

Average rate of attrition and dentin apposition of thirteen-lined ground-squirrel incisors at 21°C. (average room temperature).

Rate of attrition (mm. per week)	Upper 0.8 Lower 1.1
Rate of dentin apposition (μ per day)	12.8

3. The rate of attrition (and eruption) of the incisor was retarded in approximate proportion to the time the animal hibernated. In the control group the average rate of attrition for the upper and lower incisors respectively was 0.8 and 1.1 mm. per week (table 8). In the experimental group the average rate was: Group I (81-100% hibernation), 0.1 and 0.1 mm. per week; group II (50-71% hibernation), 0.2 and 1.0 mm. per week; and group III (0-20% hibernation), 0.4 and 2.2 mm. per week for the upper and lower incisors (table 7).

4. The rate of dentin apposition of the incisor was likewise retarded in approximate proportion to the time the animal hibernated. The average rate in the control animals was 12.8 μ per day (table 8). The calculated rate in the experimental group was: Group I (86-100% hibernation), 1.5 μ per day; group II (24-71% hibernation), 2.5 μ per day; and group III (0-20% hibernation), 6.6 μ per day (table 7).

This report demonstrates that all of the stages of tooth development, namely, growth, calcification, eruption and at-

trition, are severely retarded during hibernation. This is in accord with the marked depression of general metabolism in hibernation.

LITERATURE CITED

- ADAMS, C. O., AND B. G. SARNAT 1940 Effects of yellow phosphorus and arsenic trioxide on growing bones and growing teeth. *Arch. of Path.*, vol. 30, pp. 1192-1202.
- BENEDICT, F. G., AND R. C. LEE 1938 Hibernation and marmot physiology. Carnegie Inst. of Washington, Wash., D. C.
- BOYLE, P. E., O. A. BESSEY AND P. R. HOWE 1940 Rate of dentin formation on incisor teeth of guinea pigs on normal and on ascorbic acid-deficient diets. *Arch. Path.*, vol. 30, pp. 90-107.
- CASSENS, A. 1939 Über die beeinflussung des winterschlafs durch vitamin D₃. *Zeitschrift f. d. Gesamte Exp. Medizin*, vol. 106, pp. 521-530.
- DUBOIS, R. 1899 Physiologie de la marmotte. *J. Physiol. et Path. Generale*, vol. 1, pp. 1020-1029.
- GAGNON, J., AND M. C. PATRAS Unpublished data.
- HARRIS, H. A. 1933 Bone growth in health and disease. Oxford Univ. Press, London.
- HERZBERG, F., AND I. SCHOUR 1941 a Effects of thyroxine on rate of eruption and dentin apposition. *J. Dent. Res.*, vol. 20, p. 276.
- 1941 b The pattern of appositional growth in the incisor of the rat. *Anat. Rec.*, vol. 80, pp. 497-506.
- HOOKE, W. E., AND E. S. G. BARRON 1941 The respiration of brown adipose tissue and kidney of the hibernating and non-hibernating ground squirrel. *Am. J. Physiol.*, vol. 133, pp. 56-63.
- MASSLER, M., AND I. SCHOUR 1941 Studies in tooth development: Theories of eruption. *Am. J. Orthod. & O. S.*, vol. 27, pp. 552-576.
- SARNAT, B. G., AND I. SCHOUR 1941 and 1942 Enamel hypoplasia (chronologic enamel aplasia) in relation to systemic disease: A chronologic, morphologic and etiologic classification. Parts I and II. *J. Am. Dent. Assoc.*, vol. 28, pp. 1989-2000, and vol. 29, pp. 67-75.
- SCHOUR, I., AND H. B. VAN DYKE 1932 Changes in the teeth following hypophysectomy. I. Changes in the incisor of the white rat. *Am. J. Anat.*, vol. 50, pp. 397-433.
- SCHOUR, I., AND M. C. SMITH 1934 The histologic changes in the enamel and dentin of the rat incisors in acute and chronic experimental fluorosis. *U. of Ariz. Col. of Agri. Tech. Bull.* 52, pp. 69-91.
- SCHOUR, I., W. R. TWEEDY AND F. A. MCJUNKIN 1934 The effect of single and multiple doses of the parathyroid hormone on the calcification of the dentin of the rat incisor. *Am. J. Path.*, vol. 10, pp. 321-342.
- SCHOUR, I., AND S. R. STEADMAN 1935 The growth pattern and daily rhythm of the incisor of the rat. *Anat. Rec.*, vol. 63, pp. 325-333.
- SCHOUR, I. 1936 Changes in the incisor of the thirteen-lined ground squirrel (*Citellus tridecemlineatus*) following bilateral gonadectomy. *Anat. Rec.*, vol. 65, pp. 177-199.

- SCHOUR, I., S. B. CHANDLER AND W. R. TWEEDY 1937 Changes in teeth following parathyroidectomy. I. The effects of different periods of survival, fasting and repeated pregnancies and lactations on the incisor of the rat. *Am. J. Path.*, vol. 13, pp. 945-970.
- 1938 Calcium metabolism and teeth. *J. Am. Med. Assoc.*, vol. 110, pp. 870-877.
- SCHOUR, I., M. M. HOFFMAN, B. G. SARNAT AND M. B. ENGEL 1941 Vital staining of growing bones and teeth with alizarine red S. *J. Dent. Res.*, vol. 20, pp. 411-418.
- SCHOUR, I., M. M. HOFFMAN AND M. C. SMITH 1941 Changes in the incisor teeth of albino rats with vitamin A deficiency and the effects of replacement therapy. *Am. J. Path.*, vol. 17, pp. 529-562.
- SUOMALAINEN, P. 1938 Production of artificial hibernation. *Nature*, No. 3609, vol. 142, p. 1157.
- VALENTIN, G. 1857 Beiträge zur kenntniss des winterschlafes der murmelthiere, Untersuchungen z. Naturlehre des Menschen, vol. 2, pp. 1-55.

PLATE 1

EXPLANATION OF FIGURES

5 Photomicrograph of a longitudinal ground section of the upper left incisor of control animal no. 44. 1 and 2, effects in dentin of first and second intraperitoneal injections of alizarine red S given 21 days apart (table 4). The distance between the two lines (measured at incisal third) was $232\ \mu$. The daily rate of dentin apposition was $11.0\ \mu$. $\times 125$.

6 Photomicrograph of a longitudinal ground section of the upper left incisor of experimental animal no. 29. 1 and 2, effects in dentin of first and second intraperitoneal injections of alizarine red S given 27 days apart (table 5). The distance between the two lines (measured at incisal third) was $109\ \mu$. The first injection of alizarine red S was given 4 days before the animal was placed in the constant-temperature room at $2-5^{\circ}\text{C}$. The second injection was given 2 days after return to laboratory temperature. The calculated rate of daily dentin apposition was $1.5\ \mu$. $\times 125$.

7 Photomicrograph of a longitudinal hematoxylin- and eosin-stained section of the upper right incisor dentin of control animal no. 44. 1, calciotraumatic line (deep hematoxylin-stained stripe preceded by light eosin-stained stripe) caused by first injection of alizarine red S (fig. 5). $\times 125$.

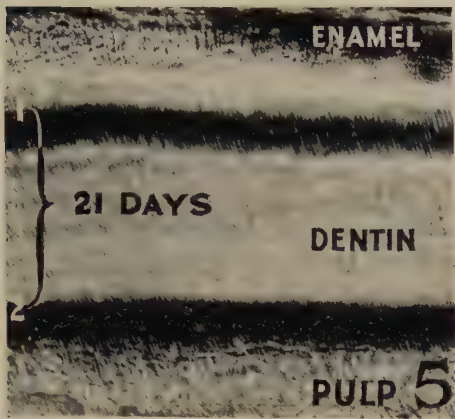
8 Photomicrograph of a longitudinal hematoxylin- and eosin-stained section of the upper right incisor dentin of experimental animal no. 29. 1, calciotraumatic line (deep hematoxylin-stained stripe preceded by light eosin-stained stripe) caused by first injection of alizarine red S (fig. 6). $\times 125$.

9 and 10 Photomicrographs of longitudinal hematoxylin- and eosin-stained sections of the upper right incisors of thirteen-lined ground-squirrels nos. 33 and 58. 1 and 2, calciotraumatic lines, effects of first and second intra-peritoneal injections of alizarine red S. $\times 125$.

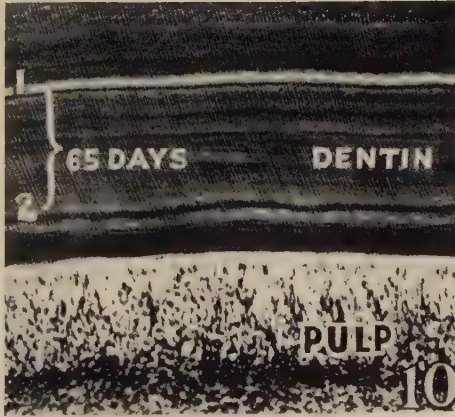
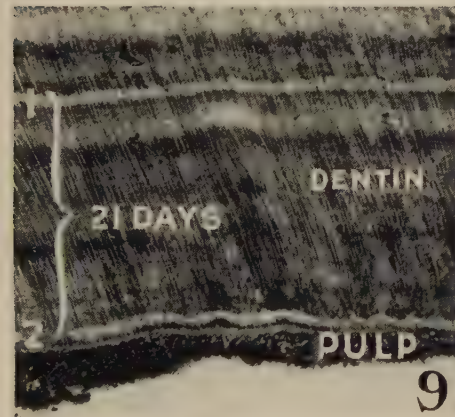
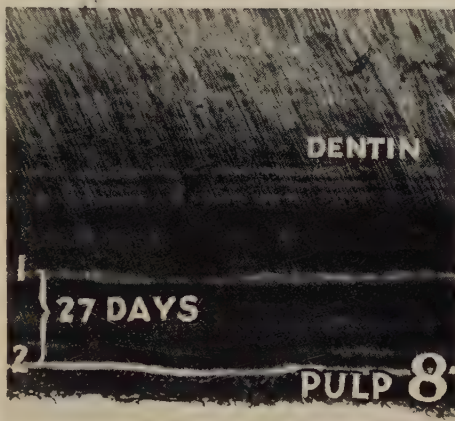
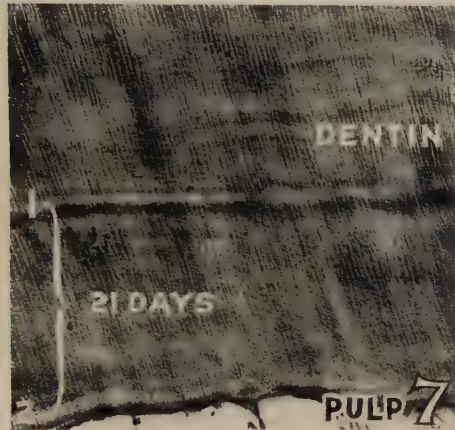
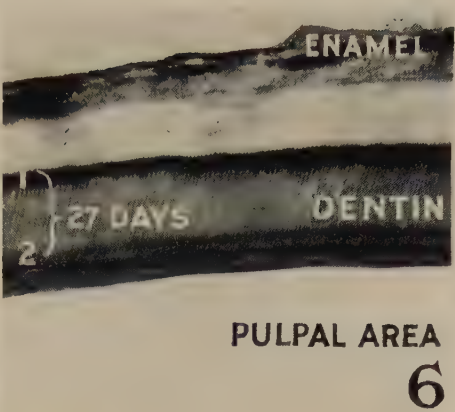
9 Animal no. 33 was kept at laboratory temperature and the time between the first and second injections of alizarine red S was 21 days.

10 Animal no. 58 was kept at $2-5^{\circ}\text{C}$. for 59 days. The injections of alizarine red S were given 65 days apart, 4 days before and 2 days after the animal was at $2-5^{\circ}\text{C}$. This animal was killed 7 days after the second injection of alizarine red S.

CONTROL



HIBERNATION



AN ANALYSIS OF THE RELATIVE GROWTH-RATES
WITHIN THE INCISOR TOOTH OF THE RAT ¹

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TWO FIGURES

Abnormally long incisor teeth occur in those species of animals in which there is a continuous production of tooth-structure and no attrition. These two conditions are frequently coexistent in the rat, and long incisor teeth are not rare. However, the specimen which is the basis of this investigation is a longer tooth than any so far described in the literature, and is so perfect in its form that it is possible to calculate mathematically the growth rate at any point on the cross section of the tooth. Since the incisor tooth is being used widely in the study of growth as affected by fever, x-rays, thyroxine, inorganic salts, and so forth, data on the normal growth become increasingly valuable and important.

The albino rat in which this incisor tooth was found was one of a colony maintained for studying the effect of diet upon the digestive tract. This rat was 25 months old when killed, and had received the following diet since weaning:

Ground whole wheat	67.5%
Casein	15.0%
Whole milk (dry, pwd.)	10.0%
Sodium chloride	1.0%
Calcium chloride	1.5%
Butter fat	5.0%

Fresh lettuce was provided three or four times each week.

¹I am indebted to Prof. H. A. Rademacher of the Department of Mathematics for a mathematical analysis of the spiral, which, however, is not included in this paper.

At the time the rat was sacrificed, it was very emaciated, sluggish in activity, and weighed 210 gm. It was able to eat only with difficulty. The final body weight was only 65% of the highest attained body weight. A photograph of the skull showing the tooth is shown in figure 1.



Fig. 1 A photograph of the skull of the rat showing the spiral form of the hypertrophied left incisor tooth. $\times 2$.

DESCRIPTION OF THE TOOTH

The upper left incisor tooth is hypertrophied in this animal and measures 73 mm. from the growing base to the occlusal surface. The shape of the tooth is that of a cork-screw, or the form of a helical spiral (Webster); however, D'Arcy Thompson prefers the term "helicoid," since there is no true deviation from the axis around which the spiral is formed. This tooth continued to increase in length after losing contact with the lower incisor, penetrated the lower lip once, and then grew as a spiral on the outside of the face of the rat. This hypertrophied tooth formed nearly two complete convolutions, or an arc subtended by 696 degrees. The dimensions of the tooth and of the spiral which was formed are given in table 1, and are shown graphically in figure 2. They were made with a micrometer. The length of the tooth of this rat offers an

TABLE 1

Measurements in millimeters of the tooth and the spiral.

	<i>mm.</i>
Length of tooth (total)	73.0
Length of extra-alveolar portion	58.0
Length of labio-lingual dimension	2.8
Length of mesio-lateral dimension	1.7
Diameter of spiral (outer surface)	12.0
Diameter of spiral (inner surface)	6.4
Pitch of spiral	6.0

opportunity for making certain deductions concerning the growth of the incisor tooth which cannot be made upon a tooth of the normal length. The growth of the tooth into the form of a spiral is the product of the pattern of growth of the cross section of the tooth, which is gradually being pushed distally. The pattern of growth will be discussed in the succeeding section.

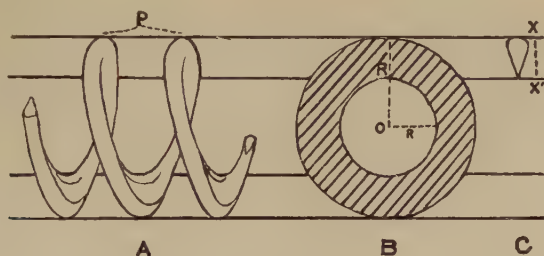


Fig. 2 A. A diagrammatic sketch of the spiral form of the tooth. P = pitch of spiral. B. An end-view of the spiral, showing inner and outer diameters of the spiral. C. A cross section of the tooth. The line X-X' is the labio-lingual axis of the tooth, and represents the direction of the growth gradient. X is place of most rapid growth; X' place of slowest growth.

DISCUSSION

The incisor teeth of the rat are said to increase in length at the rate of approximately 2 mm. per week (Addison and Appleton, '15; Schour and Steadman, '35). At that growth rate this tooth is the product of nearly 35 weeks of growth. Since this animal was relatively old, it may be that the rate of growth was somewhat slower than the rate stated above. However, there is definite proof in the perfect symmetrical

form possessed by this hypertrophied tooth that growth was continuous and orderly during the period of development. A deviation from the established pattern of growth during the 35-week period would have resulted in (1) a change in the dimensions of the cross section of the tooth, either by narrowing or enlargement, (2) a presence of a region of faulty tooth structure, which would have resulted in a breaking-off of the tooth, or (3) a departure from the true helical form. Evidence of the existence of stable growth conditions in the proliferating region is present in the constant curvature of the tooth, and the constant pitch of the spiral.

The fact that the incisor tooth forms a helical spiral when hypertrophied is proof of the existence of regions of growth in the proliferating part of the tooth which possess differential ratios in respect to each other. But the pattern of arrangement of the areas of most rapid growth, of slowest, and of intermediate rates is not as complicated as might seem to be necessary to produce a spirally-shaped tooth.

It is apparent that the spiral is the resultant effect of two factors, just as all skew curves are characterized by the fact that they exhibit a constant curvature and a constant torsion. These motions are explained in all textbooks on differential geometry (Graustein, '35) and are based on tangent and oscillating planes. Curvature is the change in direction per unit of arc; torsion is the changing of the orientation of the oscillating plane per unit of arc. A curve of torsion zero always remains in the same plane, and would result in a simple, circular tooth, and not a spiral.

The constant curvature of the incisor teeth has been considered by Addison and Appleton ('15) and Hammett and Justice ('23), as well as others. All agree that the differential growth rates of the labial and lingual borders of the tooth account for the curvature, or arc, which the tooth forms, but since there is a lateral displacement of the occlusal end of the tooth as well, it must be taken into account in measuring the relative length of the labial and lingual borders. The formula

used to determine the length of the labial surface was as follows:

$$L = \sqrt{(2\pi \times R)^2 + P^2} \quad \text{where}$$

R = radius 6.0 and P = pitch 6.0. The nature of these measurements is shown in figure 2.

Substituting the above values, it is found that a single convolution of the spiral measured along the labial surface was 38.17 mm.

The length of the lingual surface of the tooth was determined by the use of the same formula — substituting the value of $r = 3.2$ for $R = 6.0$. The length of a single convolution of the tooth measured along the lingual surface was 20.98 mm.

The ratio of the labial-surface length of the tooth to the lingual-surface length is 1.81 to 1, so that the growth rate of this tooth was 1.81 times as rapid on the labial surface as on the lingual surface. This shows the existence of a growth gradient in the direction of the long axis of the cross section of the tooth with the highest rate of growth at the labial or enamel surface, and the lowest rate at the lingual surface.

The second factor which is present and necessary to produce the spiral form is called the torsion. This, in the case of the incisor tooth, is dependent upon the angle of deviation from the mid-sagittal plane of the skull. The lateral direction of the proliferating plane of the tooth accounts for the pitch of the spiral. A change in the direction of this plane, by more rapid proliferation on either the mesial or lateral border, would result in a change in the pitch of the spiral. It is seen from the analysis of this hypertrophied tooth that even 35 weeks of the accumulated product of growth produces no more material on the mesial than on the lateral surfaces. There is no differential growth rate extending from the mesial to the lateral surface of the tooth. But points on the mesial and lateral surface of the tooth which are equally distant from point 0 in figure 2 grow at the same rate, and can be determined by simple substitution in the formula given above. The spiral form is due entirely to growth at a constant rate in a continuously changing direction.

Examination of the examples of hypertrophied incisor teeth at The Wistar Institute of Anatomy and Biology shows various degrees of curvature and torsion (or pitch). In some cases the spiral is tightly wound and in others very loosely wound. None of the specimens show the perfect spiral form as seen in the specimen shown in figure 1, since the teeth fracture easily.

The reasons for the existence of a growth gradient in the tooth should be investigated. Histological examination of the growing end of the tooth might well throw some light on this problem.

SUMMARY

1. An analysis of the form of the incisor tooth of the rat, which attained a length of 73 mm., shows the growth rate on the labial surface to be 1.81 times as rapid as on the lingual surface.

2. This ratio of growth-rate was maintained for approximately 35 weeks, as is evidenced by the constant diameter of the spiral (12 cm.).

3. There is a gradient of growth along the axis of the tooth from the labial to the lingual surface.

4. There is no gradient of growth across the tooth from the mesial to the lateral (distal) surface.

5. The pitch of the spiral is due to the change of rate of growth along the labio-lingual axis of the tooth, plus the angle of deviation from the mid-line of the skull (torsion).

LITERATURE CITED

- ADDISON, W. H. F., AND J. L. APPLETON 1915 The structure and growth of the incisor teeth of the albino rat. *J. Morph.*, v. 26, pp. 43-46.
- ARNIN, S. S., M. F. CLARKE, B. G. ANDERSON AND A. H. SMITH 1936 Changes in rats consuming diet poor in inorganic salts. *Yale J. Biol. and Med.*, v. 9, pp. 117-126.
- GARRISON, L. H. 1940 Effect of fever on rat incisor. *J. Dent. Res.*, v. 19, pp. 215-240.
- GRAUSTEIN, WILLIAM C. 1935 *Differential Geometry*. Macmillan Co., New York.
- GRÜNEBERG, H. 1937 Relations of endogenous and exogenous factors; teeth of grey lethal mouse. *J. Anat.*, v. 71, pp. 236-244.

- HAMMETT, F. S., AND ELIZABETH S. JUSTICE 1923 The geometrical symmetry of growth of the upper incisors of the albino rat. *Anat. Rec.*, v. 26, pp. 141-144.
- HAMMETT, F. S. 1922 Studies of the thyroid apparatus. VII. A differential effect of thyro-parathyroidectomy and parathyroidectomy on the incisor teeth of the albino rat. *Am. J. Phys.*, v. 62, pp. 197-220.
- HAMPP, E. G. 1940 Mineral distribution in the developing tooth. *Anat. Rec.*, v. 77, pp. 273-291.
- KARNOFSKY, D., AND E. P. CRONKITE 1939 Effect of thyroxine on eruption of teeth in new-born rats. *Proc. Soc. Biol. and Med.*, v. 40, pp. 568-570.
- SCHOUR, I., AND S. R. STEADMAN 1935 Growth pattern and daily rhythm of incisor of rat. *Anat. Rec.*, v. 63, pp. 325-333.
- 1935 Geometric construction of rat incisor. *J. Dent. Res.*, v. 16, pp. 222-223.
- SCHOUR, I., AND M. M. HOFFMAN 1939 Tooth development; 16 microns calcification rhythm in enamel and dentine from fish to man. *J. Dent. Res.*, v. 18, 91-102.
- SMITH, R. A. 1938 Effects of x-rays on developing teeth of rats. *Am. J. Orthodontics*, v. 24, pp. 428-434.

THE EFFECT OF LOCATION ON THE QUALITY OF THE HAIR AND SKIN IN THE WHITE RAT

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ONE PLATE (FIVE FIGURES)

When areas of skin were exchanged between the ventral and dorsal sides of the body (Butcher, '36, '37) in young rats, the quality of the hair on the transplants was not greatly altered. Little alteration probably occurred because the hair of the two regions was quite similar in quality, and the transplants were made at an age (22 days) when the response of the hair follicle to environmental influence was possibly limited. Seevers and Spencer ('32) observed no effects on color or quality of hair when similar transplants were made in guinea pigs 40 to 50 days old.

These observations, however, do not answer such questions as the following. Will the quality, direction of growth and arrangement of the hair be altered when skin with a widely divergent character, such as skin from the tail, is transplanted to the backs of young rats? Will the sebaceous glands and cornification of the skin be changed in such transplants? If skin from a fetal tail is grafted to the dorsum of a postnatal rat, will the modification be greater than when skin is autotransplanted on a postnatal rat?

When transplants are made from the tail to the back, the scaliness of the skin, the size and activity of the sebaceous glands, and the length and arrangement of the hairs have been modified. An account of the survival of and changes in the transplants from fetal tails to the dorsa of postnatal rats is also included in this study of conditions influencing growth.

AUTOTRANSPLANTS MADE AT THE AGE OF 22 DAYS

Hair was first removed from the back of the rat with sodium sulphide. Then, after anesthetizing the rat with ether, the tip of its tail was amputated, and the skin from the entire circumference of this amputated piece, an area about 1 cm. long and .5 cm. wide, was cut loose. An area of skin was then quickly removed from the dorsum, and the piece from the tail was anchored by interrupted stitches on the back. In most instances, the grafts were rotated 180 degrees so as to insure later identification and to study the effect on hair slope. Vaseline was applied generously two or three times daily. Twelve of the fifteen transplants survived.

Animals were sacrificed at various intervals after grafting. The grafts and skin adjacent to the origin of the graft on the tail were fixed in formaldehyde. Hairs which appeared longest under the binocular were then plucked and measured. One hundred hairs from four different grafts, which had survived for 130 days, averaged 4.4 mm., while 100 hairs from the tails adjacent to where the grafts were taken averaged 3.4 mm. in length. Since the skin in the graft was no longer subjected to the tension which existed in the tail, the skin contracted, resulting in a much thicker dermis (compare figs. 4 and 5). With the exception of a slightly thicker corneum, the strata of the epidermis in the graft remained quite similar to comparable strata in the tail. The hair assumed nearly an erect position with less stretching of the skin in the graft, and the hair follicles were closer together. The scaliness of the skin in the graft was much reduced, and usually the skin was more oily, indicating greater activity of the sebaceous glands. When a histological section of the skin from the graft (fig. 4) is compared with a section from the tail (fig. 5), it is observed that the sebaceous glands in the graft are much larger. The hair follicles in the graft took up the growth rhythm of their new environment, and produced hair in accordance with the cyclic activity of the dorsum.

AUTOTRANSPLANTS MADE SOON AFTER BIRTH

Young rats, 2 or 3 days old, were anesthetized, and the skin was transplanted from the tail to the dorsum. The piece transplanted, of course, was small, being usually about .5 cm. long. After transplanting, the grafts were vaselined three times daily to prevent drying and sloughing. Only 20% of the twenty grafts survived. This high mortality was due to the rapid growth of neighboring skin which undermined the graft before the latter was vascularized and had started growth. A greater percentage (80%) of "takes" was secured in 22-day-old rats because the skin and hair follicles of the older rat were in the resting condition (Butcher, '34) at the time of transplantation. Because of the rapid growth of the neighboring skin and little tension on the graft in these young rats, the transplant never expanded very much, the hair follicles were very close together, and the hair was very thick. Scaliness was practically lacking, and the oily condition of the hair indicated hyperactivity of the sebaceous glands. In every instance, the hair grew almost straight up, causing the hair to appear much longer than hairs on the tail.

Two rats whose skin had been transplanted when 3 days old were killed 123 days later. Another rat, transplanted at the age of 3 days, was killed 128 days later. Grafts and pieces adjacent to the origin of the graft from the tail were fixed in formaldehyd  from each of these rats.

Fifty of the hairs which appeared longest under the binocular were plucked, mounted, and measured from each graft. Similar measurements were made of fifty of the longest appearing hairs from each piece of tail. The average length of the hairs from the grafts was 4.6 mm. while the hairs from the tail averaged only 3.52 mm. The hairs from the graft were, therefore, about 31% longer. Hairs from the back, graft, and tail are shown in figure 3.

HOMOIOTRANSPLANTS MADE FROM FETUSES TO POSTNATAL RATS

To make these grafts, the hairs from the hosts' backs was removed with sodium sulphide. Pregnant rats with fetuses

of known age were then killed. A host was anesthetized and a small rectangular piece of skin was removed from the dorsum. A fetus was removed from the pregnant rat. Its tail was amputated and the skin was removed under the binocular from the amputated piece and placed on the host. Skin from 19-day-old fetuses was fastened with delicate silk interrupted stitches. Skin from younger fetuses (15 days) was held in place by laying a piece of hard rubber, .5 mm. in thickness over the transplant, then anchoring the rubber with perforated adhesive tape.

Different aged rats served as hosts, and skin was successfully transplanted from fetuses as young as 15 days. More "takes" resulted when 22-day-old rats served as hosts since their inactive skin did not crowd the graft before it became vascularized. Only 10% of the forty transplants survived, however.

The changes induced in these grafts were similar to those in autografts of 2- or 3-day-old rats. Sebaceous glands were hyperactive, there was no scaliness, and the hairs became longer than those on the tail. One hundred of the longest appearing hairs from three different grafts averaged 4.8 mm. in length, and 100 of the longest appearing hairs from the tails of the hosts averaged only 3.8 mm. In one case where a piece of 19-day-old fetal skin was transplanted to a 7-day-old host and left 93 days, hairs 7 mm. in length were produced on the transplant. The longest hairs on the tail of this host measured only 4 mm. in length. Hairs, in this instance, had therefore increased 75% in length. Thirty-seven of the longest appearing hairs from the graft averaged 5.1 mm. in length, while thirty-seven of the longest appearing hairs from the host's tail averaged only 3.7 mm. Figures 1 and 2 show hairs from the back, the graft, and the tail of this rat. The hairs from the graft are larger than the tail hairs in every respect. The medulla is longer and slightly wider.

DISCUSSION

These experiments illustrate that several changes may be induced in the skin and hair of the tail by environment or

location in the body. When skin is transplanted from the tail to the back, scaliness has been reduced while oiliness, in general, has been increased. The size of the hair has been increased on such grafts. The average length of hairs from all grafts was 4.76 mm., while the longest hairs from the various tails averaged only 3.63 mm. The hairs of the grafts were, therefore, 31% longer. The longest hairs found on any graft were 7 mm. in length, and the longest hairs on any tail measured 4.5 mm.

This increased length is probably due to better vascularization and possibly a better hormone supply since it has been shown that the quality of feathers is modified by thyroxin injections (Domm, '39).

It was thought that the increased length of the hairs on the graft might just be due to their reaching a greater length earlier in life. To test this hypothesis 100 of the longest appearing hairs from tails of various rats, 500 days in age, averaged only 3.9 mm. in length which is not much greater than the average found for the tail hairs of the various hosts (3.63 mm.) in these experiments.

The decrease in scaliness of the skin on the graft, and the increase in activity of the sebaceous glands is a typical adjustment, and is exemplified in the evolution of amphibia where it is considered that multicellular glands displace scales (Walter, '39).

A survey of the surviving grafts shows that the hair tended to stand straight up. Even when autotransplants were made on 22-day-old rats, the hair grew nearly erect. The hair on a few of the grafts exhibited a slight tendency toward its original direction while the hair on an equal number conformed slightly to the new environment. These results are similar to those found in the guinea pig by Seevers and Spencer ('32). Trotter and Dawson ('32) rotated skin on newborn rats and found that the hair slope was a ventrocaudad or a mixed one. They concluded that the "hair slope observed in the adult is determined by the factors of tension effective at the time of development of the hair follicle." With little or no tension

on the grafts in their new location in these experiments, the skin contracted and the hair became erect. It would, therefore, seem that the slope depends as much upon continual tension as on tension just at the time of the development of the hair follicle.

The type of hair in the tail is determined prior to the fifteenth day of fetal life since hair on the transplants taken from fetal tails maintained the quality typical of the tail.

SUMMARY

1. The length of the hair was altered when skin was transplanted from the tail to the back in young rats or from fetal tails to the backs of young rats.

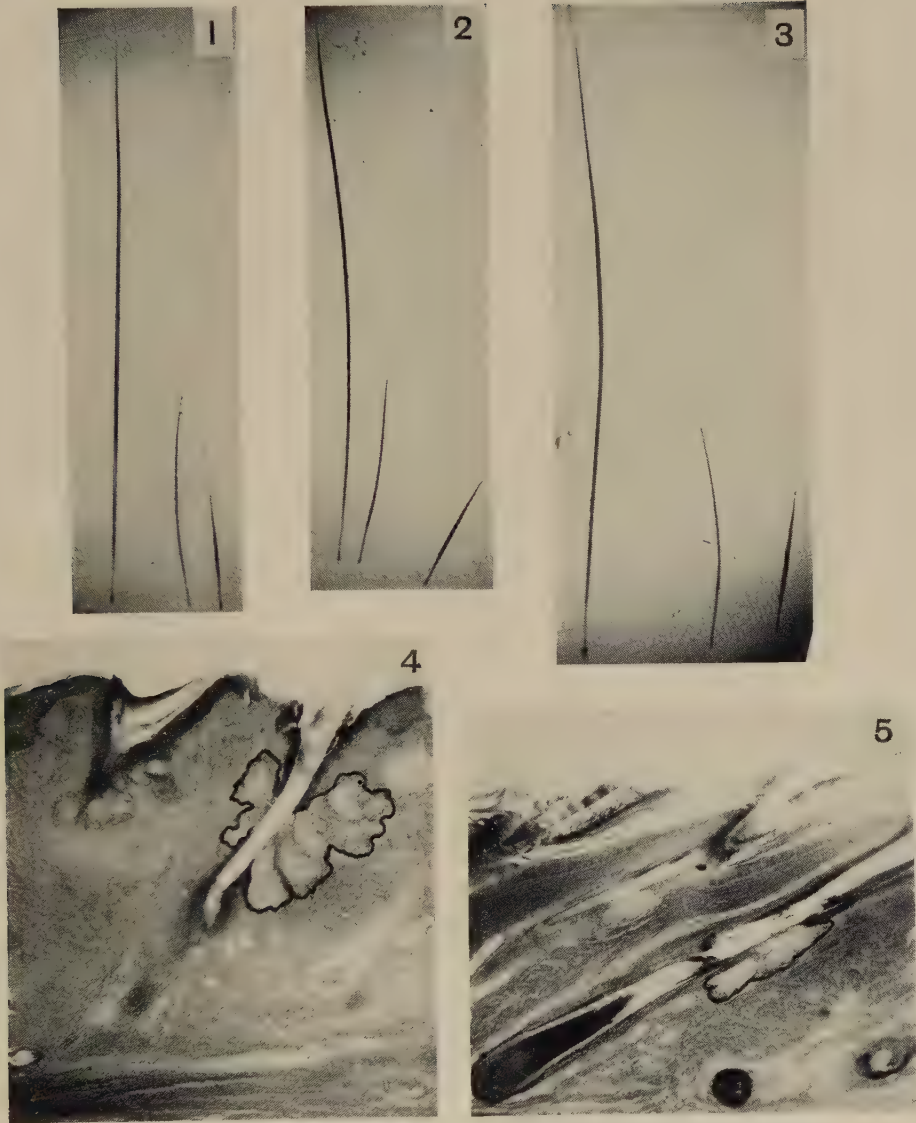
2. The length of the hair on the grafts increased as much as 75% in some cases and averaged 31% in all cases. Length can, therefore, be altered by environment.

3. Scaliness of the skin was greatly reduced on the tail grafts to the back, and there was usually enlargement and hyperactivity of the sebaceous glands.

4. The hair grew erect on the graft in most cases, indicating that continual tension is necessary for the retention of hair slope.

LITERATURE CITED

- BUTCHER, EARL O. 1934 The hair cycles in the albino rat. *Anat. Rec.*, vol. 61, pp. 5-19.
- 1936 Hair growth on skin transplants in the immature albino rat. *Anat. Rec.*, vol. 64, pp. 161-171.
- 1937 The fate and activity of autografts and homografts of skin in white rats. *Arch. Dermatology and Syphilology*, vol. 36, pp. 53-57.
- DOMM, L. V. 1939 Sex and Internal Secretions, pp. 304. Williams & Wilkins Company, Baltimore.
- SEEVERS, C. H., AND D. A. SPENCER 1932 Autoplastic transplantation of guinea pig skin between regions with different characters. *Am. Naturalist*, vol. 66, pp. 183-189.
- TROTTER, MILDRED, AND H. L. DAWSON 1932 The direction of hair after rotation of skin in the newborn albino rat: a second experiment on hair slope. *Anat. Rec.*, vol. 53, pp. 19-31.
- WALTER, H. E. 1939 Biology of the vertebrates, p. 212. The Macmillan Company, New York.



1 and 2. Hairs from back, graft, and tail (left to right in photograph). Skin from 19-day-old fetal tail was placed on the dorsum of 7-day-old rat and left 93 days. $\times 5$.

3. Hairs from back, graft, and tail. Autotransplant from tail to dorsum at age of 3 days. Left 128 days after transplanting. $\times 5.5$.

4. Autotransplant made at age of 21 days and left for 56 days. Compare with figure 5 and note erectness of hair, and enlargement of sebaceous gland. $\times 30$.

5. Skin from tail adjacent to origin of graft shown in figure 4. $\times 30$.

A CASE OF PARALLEL EMBRYONIC DEVELOPMENT IN THE RAT AND ITS BEARING ON THE QUESTION OF SUPERFETATION ¹

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ONE PLATE (FIVE FIGURES)

The alleged phenomenon of superfetation, or the occurrence of a second conception in a pregnant mammal, has interested writers and investigators since the time of Aristotle. Two general types of observations have been made in several species of mammals. The first includes cases in which fetuses of two markedly different sizes were delivered or found in the uterus (Jepson, 1883; Harman, '17; Hunt, '19; Marshall, '22). The death of the smaller fetuses in utero and their failure to resorb, may account for these findings. Explanations on the basis of superfetation are unwarranted (Kuntz, '16, '20; Meyer, '19). In the second category are included reports of animals which delivered two litters of living young at intervals (King, '13; Sumner, '16; Smith, '27 a, b; Slonaker, '34; Markee and Hinsey, '35; Stowell, '41). Markee and Hinsey ('35) suggest that, as an alternative to superfetation in the latter cases, "all of the young may develop from one period of ovulation and fertilization, and that some of the young are retained in the uterus for an abnormally long period."

The present paper deals with a case of parallel development of two sets of embryos in the uterus of a rat. Without knowledge of the detailed history of the animal it might have been cited as an illustration of superfetation. Since most of

¹This investigation was supported in part by a grant from the Committee for Research in Problems of Sex, National Research Council.

the facts are known, however, the unusual conditions observed are subject to an entirely different interpretation which may help to explain the cases of so-called superfetation which abound in the literature.

REPORT OF CASE

A virgin female rat (D 419), $3\frac{1}{2}$ months old, was inseminated on February 19, 1940. On March 10th, she was placed with a male in a separate cage and on the following day, the twenty-second day of pregnancy, delivered a normal litter of nine. Spermatozoa were found in the vagina the following morning and the male was removed from the cage. The female was isolated with her nine young for 28 days and then sacrificed. Her reproductive organs were removed and fixed in Bouin's solution.

Three implantation sites were observed in the right uterine cornu and five in the left (fig. 1). One of the three on the right side was large and two were small, showing external indications of resorption. On the left side four implantation sites were of medium size and appeared normal and one, at the lower end, appeared to be resorbing. The embryo was removed from the large implantation site in the right cornu. Comparison with a series of normal embryos showed it to be at the same stage of development as a normal $15\frac{1}{2}$ -day embryo (fig. 2). One of the embryos in the left cornu was removed and compared with the normal series. It was at the same stage of development as a normal 11-day embryo (fig. 3). The remaining implantation sites were serially sectioned. Examination of the slides confirmed the observation that three of the embryos were resorbing and that four, all at the same stage of development, were growing normally (figs. 4 and 5). Thus the uterus contained four embryos at the same stage of development as an 11-day normal embryo, one equal to a $15\frac{1}{2}$ -day normal embryo and three resorption areas.

By assuming that post-implantation development was normal (Enzmann, Saphir and Pincus, '32) it was possible to determine the dates of implantation by subtracting the

estimated ages of the embryos from the actual age (28 days) and adding the number 6 (the length of the normal pre-implantation period). Thus the large embryo must have implanted on day $18\frac{1}{2}$ and the smaller ones on day 23 ($4\frac{1}{2}$ days later).

Examination of the ovaries revealed that there were five corpora lutea of uniform size in the left ovary and six in the right. After the tissues had been run up to 80% alcohol, the corpora lutea were dissected out and their diameters measured with a Vernier caliper under a binocular dissecting microscope. The average diameter was 1.768 mm.

DISCUSSION *

It is generally agreed that the length of life of spermatozoa in the reproductive tract of the female rat or mouse is short (Yochem, '29; Hartman, '32, '39; Lewis and Wright, '35; Merton, '39). Since the date of insemination is known in the case reported here, and since the male was removed from the cage the morning after insemination, it is safe to assume that all embryos resulted from one ovulation and fertilization. The obvious explanation for the discrepancy in size of the two sets of embryos, is that they implanted at different times ($4\frac{1}{2}$ days apart).

Kirkham ('16 a, b, '18) and Enzmann, Saphir and Pincus ('32) have shown that in inseminated lactating rats and mice there is a delay in implantation, correlated in general with the number of suckling young. The latter investigators report that once implantation has occurred the growth curve of the developing young is indistinguishable from that of embryos during normal pregnancy. Krehbiel ('41 a) states that there are various times at which implantation may take place in pregnant lactating rats. The periods occur at intervals of every 4 or 5 days and coincide with periods of follicular growth during pregnancy as reported by Swezy and Evans ('30). It would seem that in the case of delayed implantation described here, one of the fertilized eggs implanted on day $18\frac{1}{2}$ and the

others remained free in the lumen of the uterus until day 23 when they in turn implanted.

The author feels that the explanation is to be sought in either general or local variations in the estrogen content of the maternal blood stream. Krehbiel ('41 b) and Weichert ('41, '42) have reported that small amounts of estrogen injected into pregnant lactating rats will bring about implantation of blastodermic vesicles which would otherwise lie free in the uterine lumen for many more days. It is possible that in the rat reported here, an increase in the estrogen content of a localized area of the uterus was responsible for bringing about the implantation of the larger embryo on day 18½. The close proximity of this embryo to the right ovary may be of significance in this connection. By day 23 the general estrogen level may have been high enough to bring about implantation of the remaining embryos.

After implantation has occurred in the pregnant lactating rat, the growth curve of the corpora lutea follows that of normal pregnancy (Weichert and Schurgast, '42). From examination of the embryos of this animal it would be expected, therefore, that the corpora lutea would be similar in size to those of a normal rat on the fifteenth day of pregnancy. However, the diameter of the corpora lutea (1.768 mm.) while greater than in a normal animal on the eleventh day (1.503 mm.), is less than on the fifteenth day (1.907 mm.). A clear explanation of this discrepancy is not as yet available.

Workers who have reported cases of so-called superfetation, have assumed that a second ovulation and fertilization occurred during pregnancy. There is some basis for such an assumption. It has been demonstrated that ovulation can be induced in pregnant animals by injecting gonadotrophic substances of pituitary or placental origin (Friedman, '29; Snyder and Wislocki, '31; Wislocki and Snyder, '31; Wolfe, '31; Hill and Parkes, '32; Snyder, '34). It has likewise been observed that estrus and copulation may occur during pregnancy (Long and Evans, '22; Marshall, '22; Nelson, '29; Crew and Mirskaia, '30; Watt, '31). However, no workers have shown that both

ovulation and copulation may occur in the same animal during pregnancy except Watt ('31) whose report on the mouse is scarcely to the point since both ovulation and copulation occurred on the twenty-second day of pregnancy and parturition was delayed. The author has likewise observed spermatozoa in the vaginas of 2 rats (D 278; D 444) on the twenty-second and twenty-third days of pregnancy. Both animals experienced difficulty in delivering their young.

The experimental production of superfetation in the rabbit reported by Wislocki and Snyder ('31), was carried out under extremely abnormal conditions. Ovulation was induced during pregnancy by injecting anterior lobe extract and the animal was artificially inseminated. Upon autopsy, after only 1 day of development, fertilized ova were found. Whether the fertilized eggs would have implanted and developed further was not demonstrated.

In the case reported here, where implantation of ova occurred at different times, the rat was undergoing a prolonged lactation pregnancy. It is not unreasonable to suppose that a similar phenomenon might occasionally occur in a non-lactating pregnant animal.

In cases of supposed superfetation, workers have been at a loss to explain how the older fetuses could pass the second set of implantation sites at the time of delivery without dislodging the younger embryos. In the present case, had the embryos gone to term, no such difficulty would have been encountered since the embryos of each size were confined to their respective cornua.

Thus, contrary to the opinion of many investigators, it seems possible that a second conception during pregnancy never occurs. Cases of "superfetation" may just as logically be explained on the basis of variation in time of implantation among the same set of fertilized eggs.

SUMMARY

A case of parallel development of two sets of embryos in the uterus of a rat has been described. The animal, suckling

nine young, was inseminated during the post-partum estrus after which the male was removed. Upon autopsy on the twenty-eighth day of pregnancy, two sizes of embryos, $4\frac{1}{2}$ days apart in age, were present. The ovaries showed that only one ovulation had occurred. The bearing of this report on the question of superfetation is discussed.

LITERATURE CITED

- CREW, F. A. E., AND L. MIRSKAIA 1930 Mating during pregnancy in the mouse. *Nature*, vol. 125, p. 564.
- ENZMANN, E. V., N. R. SAPHIR AND G. PINCUS 1932 Delayed pregnancy in mice. *Anat. Rec.*, vol. 54, p. 325.
- FRIEDMAN, M. H. 1929 Mechanism of ovulation in the rabbit. II. Ovulation produced by the injection of urine from pregnant women. *Am. J. Physiol.*, vol. 90, p. 617.
- HARMAN, MARY T. 1917 A case of superfetation in the cat. *Anat. Rec.*, vol. 13, p. 145.
- HARTMAN, C. G. 1932 Ch. XIV. Allen—"Sex and Internal Secretions" 1st edition. Williams and Wilkins, Baltimore.
- 1939 Ch. IX. Allen—"Sex and Internal Secretions" 2nd edition. Williams and Wilkins, Baltimore.
- HILL, M., AND A. S. PARKES 1932 The relation between the anterior pituitary body and the gonads. Part IV. Induction of ovulation during pregnancy and its effect on the fetuses. *Proc. Roy. Soc. of London, Series B*, vol. 110, p. 180.
- HUNT, H. R. 1919 Birth of two unequally developed cat fetuses (*Felis domestica*). *Anat. Rec.*, vol. 16, p. 371.
- JEPSON, S. L. 1883 A case of superfetation in the cat. *Am. J. Obst.*, vol. 16, p. 1056.
- KING, HELEN D. 1913 Some anomalies in the gestation of the albino rat (*Mus norvegicus albinus*). *Biol. Bull.*, vol. 24, p. 377.
- KIRKHAM, W. B. 1916 a The prolonged gestation period in nursing mice. *Anat. Rec.*, vol. 10, p. 219.
- 1916 b The prolonged gestation period in suckling mice. *Anat. Rec.*, vol. 11, p. 31.
- 1918 Observation on the relation between suckling and the rate of embryonic development in mice. *J. Exp. Zool.*, vol. 27, p. 49.
- KREHBIEL, R. H. 1941 a The effects of lactation on the implantation of a concurrent pregnancy in the rat. *Anat. Rec.*, vol. 81, p. 43.
- 1941 b The effects of theelin on delayed implantation in the pregnant lactating rat. *Anat. Rec.*, vol. 81, p. 381.
- KUNTZ, A. 1916 A note on superfetation. *Interstate Med. J.*, vol. 23, p. 17.
- 1920 Retention of dead fetuses in utero and its bearing on the problems of superfetation and superfecundation. *Anat. Rec.*, vol. 18, p. 295.

- LEWIS, W. H., AND E. S. WRIGHT 1935 On the early development of the mouse egg. Carnegie Inst. Wash., Pub. 459, p. 113.
- LONG, J. A., AND H. M. EVANS 1922 The oestrous cycle in the rat and its associated phenomena. Mem. Univ. Cal., no. 6, p. 1.
- MARKEE, J. E., AND J. C. HINSEY 1935 A case of probable superfetation in the cat. Anat. Rec., vol. 61, p. 241.
- MARSHALL, F. H. A. 1922 The Physiology of Reproduction. London, p. 154.
- MERTON, H. 1939 Studies on reproduction in the albino mouse. III. The duration of life spermatozoa in the female reproductive tract. Proc. Roy. Soc. Edin., vol. 59, p. 207.
- MEYER, A. W. 1919 The occurrence of superfetation. J. A. M. A., vol. 72, p. 769.
- NELSON, W. O. 1929 Oestrus during pregnancy. Science, vol. 70, p. 453.
- SLONAKER, J. R. 1934 Superfetation in the albino rat. Amer. J. Physiol., vol. 108, p. 322.
- SMITH, A. D. B. 1927 a A case of superfetation in the pig. J. Anat., vol. 61, p. 329.
- 1927 b Further cases of superfetation in pigs and sheep. J. Anat., vol. 62, p. 100.
- SNYDER, F. F. 1934 The prolongation of pregnancy and complications of parturition in the rabbit following induction of ovulation near term. Johns Hopkins Hosp. Bull., vol. 54, p. 1.
- SNYDER, F. F., AND G. B. WISLOCKI 1931 Further observations upon the experimental production of ovulation in the rabbit. Johns Hopkins Hosp. Bull., vol. 49, p. 106.
- STOWELL, R. E. 1941 A case of probable superfetation in a mouse. Anat. Rec., vol. 81, p. 215.
- SUMNER, F. B. 1916 Notes on superfetation and deferred fertilization among mice. Biol. Bull., vol. 30, p. 271.
- SWEZY, O., AND H. M. EVANS 1930 Ovarian changes during pregnancy in the rat. Science, vol. 71, p. 46.
- WATT, LUCY J. 1931 Ovulation, oestrus and copulation with consequent dystocia during pregnancy in the mouse. Science, vol. 73, p. 75.
- WEICHERT, C. K. 1941 The effectiveness of estrogen in shortening delayed pregnancy in the rat. Anat. Rec., vol. 81, supp. p. 28.
- 1942 The experimental control of prolonged pregnancy in the lactating rat by means of estrogen. Anat. Rec., vol. 83, p. 1.
- WEICHERT, C. K., AND A. W. SCHURGAST 1942 Variations in size of corpora lutea in the albino rat under normal and experimental conditions. Anat. Rec., vol. 83, p. 321.
- WISLOCKI, G. B., AND F. F. SNYDER 1931 On the experimental production of superfetation. Johns Hopkins Hosp. Bull., vol. 49, p. 103.
- WOLFE, J. M. 1931 Ovulation in the pregnant rabbit. Anat. Rec., vol. 49, p. 191.
- YOCHER, D. E. 1929 Spermatozoön life in the female reproductive tract of the guinea pig and rat. Biol. Bull., vol. 56, p. 274.

PLATE 1

EXPLANATION OF FIGURES

1 Ventral view of reproductive tract of rat D 419. One large implantation site and two resorption areas are in right cornu. Four medium sized implantation sites and one resorption area are in left cornu.

2 a Normal rat embryo on fifteenth day of development. A normal 11-day embryo at the same magnification is shown below.

2 b Large embryo removed from right cornu of D 419. One of the embryos removed from the left cornu and at the same magnification, is shown below.

3 a Normal rat embryo on eleventh day of development (highly magnified).

3 b One of embryos removed from left cornu of D 419 (highly magnified).

4 Section of uterus of D 419 through resorption area in left cornu.

5 Section of uterus of D 419 through an implantation site in left cornu.



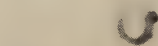
1



2A



2B



3A



3B



4



5

A COMPARISON OF THE POSTERIOR BOUNDARIES OF LUNGS AND PLEURA AS DEMONSTRATED ON THE CADAVER AND ON THE ROENTGENOGRAM OF THE LIVING ¹

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EIGHT FIGURES

INTRODUCTION

Our knowledge of pleural and pulmonary boundaries as presented in standard anatomical treatises is based on dissections of preserved cadavers. More recent investigations have attempted to re-examine these boundaries by radiographic approach in the living. Accordingly it seems worth-while to compare the results of these two methods of exploration. Complete conformity of results can hardly be expected if one keeps the following facts in mind: (1) In the cadaver all muscles are either in a state of complete relaxation or are abnormally rigid due to rigor mortis, there being some displacement of the viscera in either case. (2) The fat is solidified, not fluid as it is at body temperature in the living. (3) The cadaver is explored in the supine position with the thorax in a state of expiration, the viscera thus being displaced dorsally by gravitation. (4) The connective tissue is oedematous due to diffusion of embalming fluid into the tissue spaces, and the veins are for the most part desanguinated. (5) The position of the viscera in the cadaver is explored after the thoracic

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Drawings nos. 3, 4, 7 and 8 were made from roentgenograms in the files of the X-Ray Department of the University Hospital. All figures were drawn by Miss Sue Browder, Graduate Assistant in the Department of Anatomy.

and abdominal cavities have been opened, with resulting non-physiologic equalization of pressure.

These pitfalls are avoided by radiographic study of the living, a study which can often be made with the subject in the upright position and which, when undertaken by an experienced examiner, is capable of furnishing correct and valuable information on visceral topography.

Surprisingly enough the results of radiographic investigation of the pleural and pulmonary boundaries, although available in the roentgenographic literature, have as yet played little part in our teaching of this important chapter of gross anatomy, which is still almost exclusively based on cadaver studies. Since as a rule only the posterior mediastinal boundaries and the posterior inferior extent of lungs and pleura can be investigated on the roentgen film, the present paper will be limited to a discussion of these boundaries.

A. THE POSTERIOR MEDIASTINAL BOUNDARIES OF LUNGS AND PLEURA

Conventional anatomical teaching

According to anatomical texts the posterior costal pleura is continuous with the mediastinal pleura along a vertical line on the lateral aspect of the spinal column. This line of pleural reflection extends from the first to the twelfth thoracic vertebra, where it becomes continuous with the diaphragmatic reflection. The line of junction of the posterior costal and mediastinal pleurae is not sharply delineated; rather is it represented by a gentle curve (Morris, '33; Cunningham, '37). The customary pleural surface projection of these boundaries is shown in figure 1 a. The extent of the lungs in this region is said to coincide with the line of pleural reflection just described. The postero-medial margins of lungs and pleura define also the shape and contours of the posterior portion of the mediastinum. This is consequently regarded as a rather wide space, in which we find the esophagus in close contact with the anterior surface of the spinal column, a relationship confirmed by conventional cross sections (fig. 2 a).

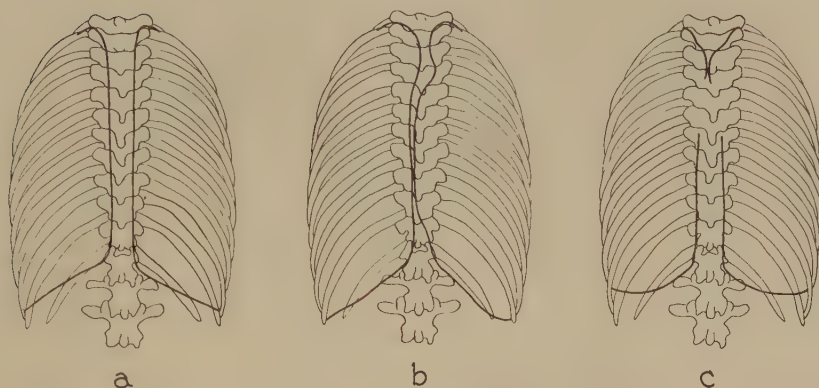


Fig. 1 Surface projections of the posterior pleural boundaries: a. According to conventional anatomical description; b. According to Heiss' findings arrived at by special dissection; c. According to roentgenograms of the living subject.

Roentgenographic findings

Only under favorable conditions of radiographic exposure, i.e., on films taken with high roentgenographic penetration and preferably with Bucky diaphragm, is it at all possible to identify the upper medial lung portions as a more translucent area superimposed on the shadow of the spinal column (Danelius, '29 and '31; Arendt, '33; Bársony and Wald, '36; Stéphan and Kirsch, '36; Maier, '40). The medial contour of this translucency describes a convex arc which arises in the apical field and continues downward and medially in or close to the midline. At the level of the fourth or fifth thoracic vertebra the lung borders of the two sides come almost in contact leaving but a small interval between them. In addition to the pulmonary translucency the corresponding pleural reflections of the two sides may appear as thin lines visible within the tracheal translucency (fig. 3). The area thus outlined can be definitely attributed to posterior medial regions of lungs and pleura, not to anterior portions, as erroneously stated in the literature (Fanconi, '31; Wechsler, '31; Ottonello, '32 a). Since the pulmonary translucencies in question reach the midline above the level of the jugular notch of the sternum, they cannot be produced by the medial anterior margins of

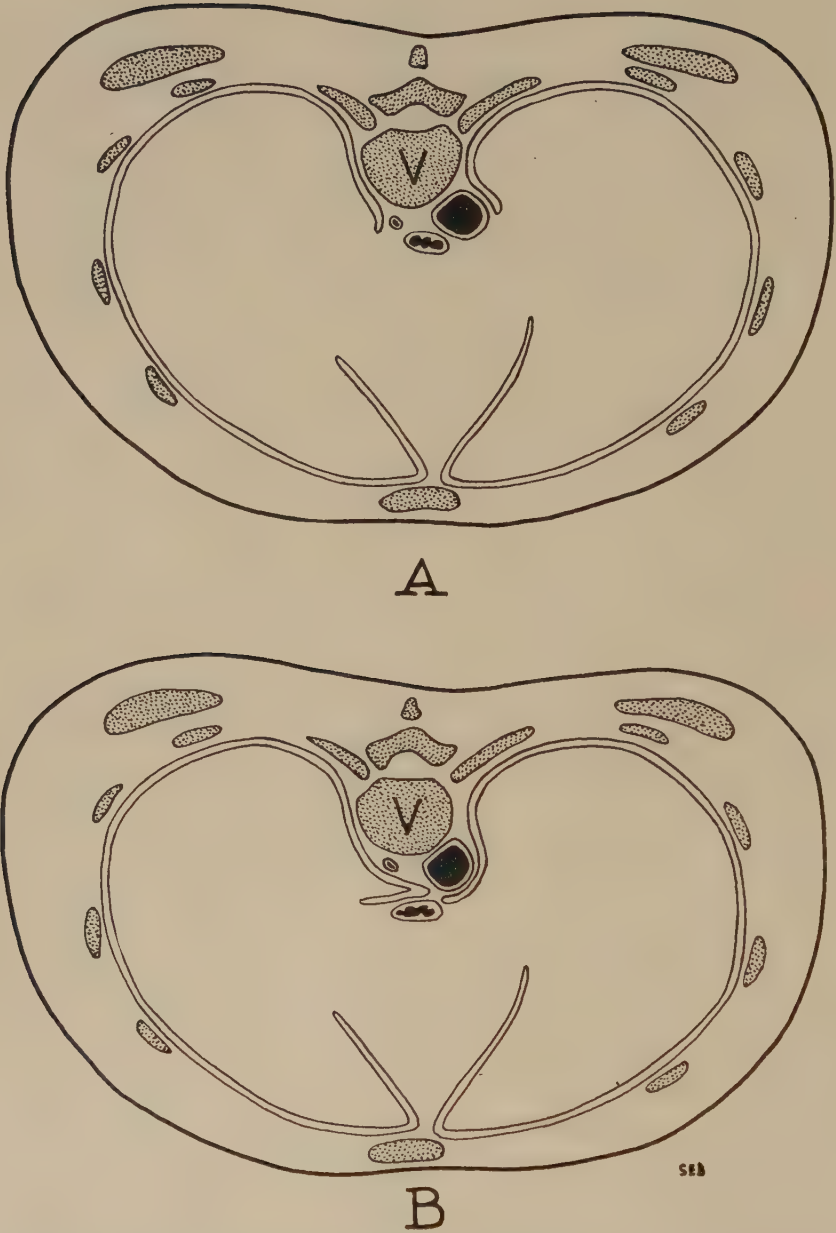


Fig. 2 Cross section through the posterior mediastinum at the level of the fifth thoracic vertebra. a. Conventional diagram. b. Modified diagram incorporating x-ray findings.

the lungs, which at this level have not yet reached the mid-line and are farther apart from each other (Danelius, '33). In stereo-roentgenographic investigations in the living, after the esophagus was filled with a radiopaque contrast medium, Pratje ('24) was able to demonstrate that the distance between esophagus and spinal column at the level of the fourth and fifth thoracic vertebra amounts to 2-4 cm. This space is occupied by pleural recesses into which medial parts of the lungs extend. According to this author the pleural folds approach

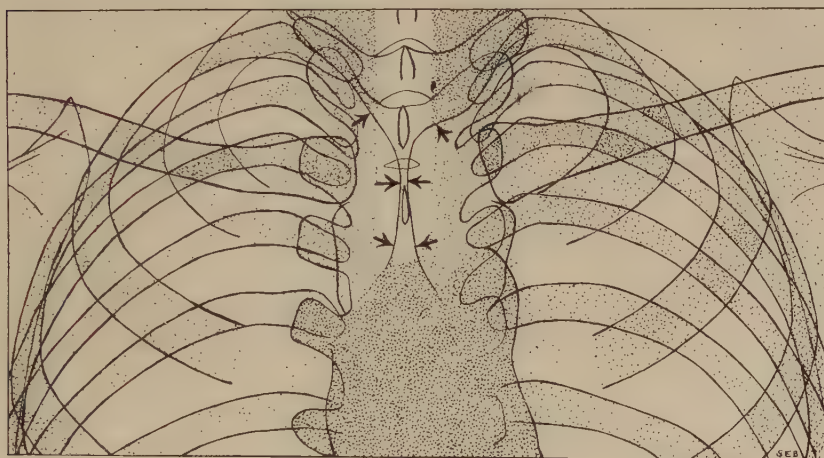


Fig. 3 Diagram taken from roentgenograms depicting the presence of lung in the retro-oesophageal space as an increased translucency being superimposed over the shadow of the vertebral column. Arrows indicate pleural mediastinal boundaries.

each other so closely behind the esophagus that only a thin layer of connective tissue lies between them. Therefore they can rightly be regarded as the mesentery of the esophagus. In roentgenographic studies on the cadaver and in the living, Danelius confirmed the presence of a retro-esophageal space, which is occupied by lungs and pleura. This space deepens during inspiration and in the upright position, while it becomes more shallow during expiration and in the supine posture (Pratje, '24; Danelius, '31). Also the space is larger in cases

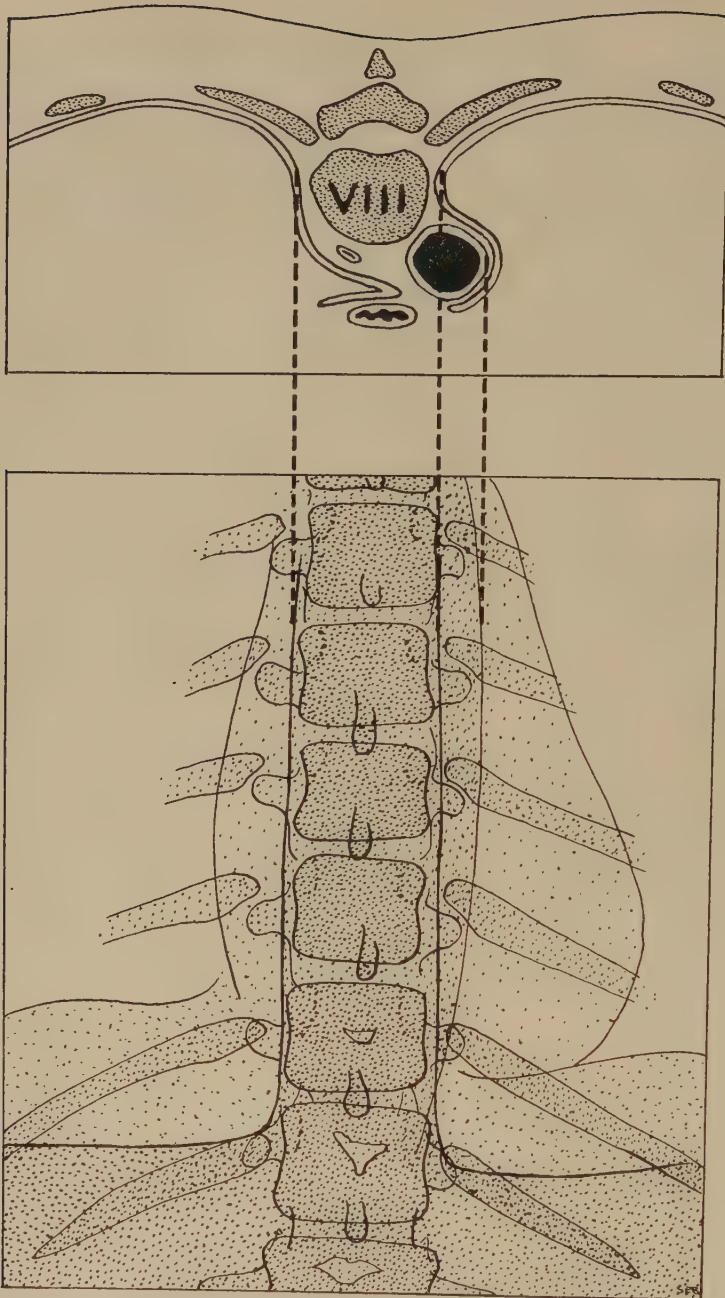


Fig. 4 Upper: Cross section through posterior mediastinum at level of eighth thoracic vertebra. Lower: Diagram taken from roentgenogram depicting posterior portions of visceral and/or parietal pleura as lines accompanying the vertebral column. Dotted lines indicate anatomical substrate of pleural lines and aortic shadow in cross section.

where the thoracic curvature is pronounced, or where there is a distinct thoracic kyphosis, and it may be absent if this curve is ill defined (Bársony and Wald, '36).

Possibility of the presence of lung and pleura in this retro-esophageal location is greater on the right side, indeed, the right mediastinal pleura frequently encroaches on the territory of the left pleura by crossing the midline.

From the level of the tracheal bifurcation downward over several vertebrae the medial boundaries of lungs and pleura become indefinite on the roentgenogram (fig. 1 c). In the lower thoracic region they frequently reappear, more commonly on the left than on the right, as longitudinal linear shadows closely paralleling the lateral border of the spinal column down to the diaphragmatic reflection (fig. 1 c and fig. 4). These lines correspond to posterior portions of visceral and/or parietal pleura, which are struck tangentially by the x-ray beam and are contrasted against the adjacent air-containing lung regions. The frequent absence of these pleural shadows on properly exposed x-ray films is due to the fact that only insufficient portions of the pleura lie in a plane parallel to the central x-ray beam. Their density is then not sufficient to register on the film. However, their presence on the roentgenogram does not prove that the mediastinal margins of lungs and pleura are located in a sagittal plane throughout the whole antero-posterior extent of the mediastinum from spinal column to sternum, a matter to be discussed later.

Older continental literature

Whereas with one exception² no mention of the retro-esophageal location of lungs and pleura could be found in standard American or English anatomical texts, the older continental literature discusses these relations in detail.

² Grant ('40) mentions the pleural approximation in the posterior mediastinum and illustrates by diagram the presence of a meso-esophagus, but other of his figures contradict this, and his text on the anatomy and relations in the posterior portion of the superior mediastinum takes no account of the pleural culs-de-sac and the lungs contained in them.

Poirier and Charpy ('01) state explicitly in their textbook that the mediastinal surface of each lung does not lie in a straight antero-posterior plane, but insinuates itself, covered by mediastinal pleura, between the organs of the posterior mediastinum. The authors describe four culs-de-sac of mediastinal pleura occasionally encountered by them, two on the right and two on the left. The most constant is the right pleural duplication behind the esophagus and in front of the azygos vein. It is represented by a very distinct out-pouching of the mediastinal pleura which the authors call "cul-de-sac pleural inter-azygo-oesophagien" (fig. 5). A similar cul-de-sac makes its appearance on the left between aorta and esophagus. Other culs-de-sac in front of the esophagus are less distinct and not so constant. Surprisingly enough the French authors concede the presence of these pleural pockets only at levels inferior to the arch of the azygos vein on the right and the aortic arch on the left, and state that above these points the culs-de-sac are not demonstrable. Our own radiographic observations have detected their presence particularly in the upper mediastinum. Testut ('05) likewise discusses the presence of these culs-de-sac. According to him a right mediastinal recess is especially well marked. It is located between the vertebral column and esophagus in the lower part of the mediastinum. Braune, a German classical anatomist, also depicts this pleural outpouching behind the esophagus in a cross section which is reproduced by Spalteholz (Hand Atlas, 7th edition, vol. 3, fig. 683), although he does not mention this arrangement in his text. Merkel ('02) is very explicit about the anatomical relations of the pleura in the region under discussion. He points out that the pleura, after its reflection from the costal surface onto the spinal column, forms a gutter which is more distinct on the right than on the left. This pleural recess contains a ridge of lung tissue, the crista pulmonis. Merkel apparently was the first to describe this pulmonary ridge and place it correctly in its relation to the pleural cul-de-sac. Figures of the medial surfaces of both right and left lungs show these ridges well marked also in the

superior portions. In this connection Merkel observes that due to the pulmonary ridges the posterior region of the mediastinum directly in front of the spinal column is rather narrow from side to side.

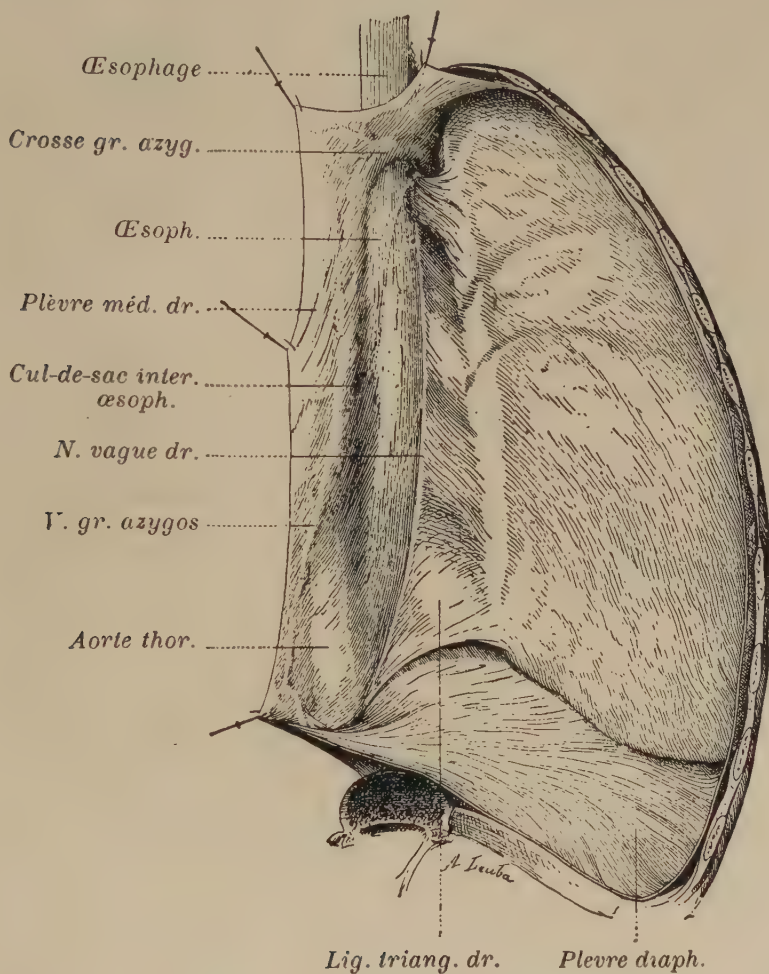


Fig. 5. Reproduction of a figure in Poirier and Charpy on relations of right mediastinal pleura to esophagus, azygos vein, and thoracic aorta in the new born. The right pleural sac has been widely opened from behind. Mediastinal portion of parietal pleura has been drawn to the left. Note legend calling attention to retro-esophageal cul-de-sac.

Heiss ('19) undertook very careful investigations on the posterior pleural boundaries of the mediastinum. By injecting a colored, solidifying material into the pleural cavity of cadavers and following dissection from a posterior approach he explored the limits of the posterior mediastinum and demonstrated the mediastinal reflections of the pleura. He found that the mediastinal pleural borders approach each other closely in the upper mediastinum posterior to the esophagus. The vascular arches of aorta and azygos vein produce indentations of the pleural contours, the indentation on the right being located at the level of the second thoracic vertebra. Caudal to this level the right pleural cavity extends to the left of the midline until at the level of the fifth thoracic vertebra it is only 1 cm. removed from the left pleural cavity. This retro-esophageal recess of the right pleural cavity encroaches upon the left side as far down as the tenth thoracic vertebra. The aorta partly overlaps the right pleural cul-de-sac posteriorly (fig. 6). On the left side the pleural mediastinal septum follows in general the lateral border of the spinal column, but approaches more closely the midline in its highest portion. Figure 1 b illustrates the surface projection of these pleural boundaries according to Heiss.

Discussion

From the facts presented above it is evident that topographical relations which were well known to older generations of anatomists but which were for some reason disregarded by writers of our current textbooks, have now been rediscovered and revitalized by roentgenographic approach. In the light of these studies we must revise our concept of the posterior mediastinal boundaries of lung and pleura. This is illustrated in figure 1, which contrasts the surface projection of the posterior pleural margins according to conventional anatomical descriptions (fig. 1 a) with the results of Heiss' special investigations on cadavers (fig. 1 b) and with recent x-ray studies in the living (fig. 1 c). Also very instructive in this connection is a comparison of cross sections illustrating the two concepts.

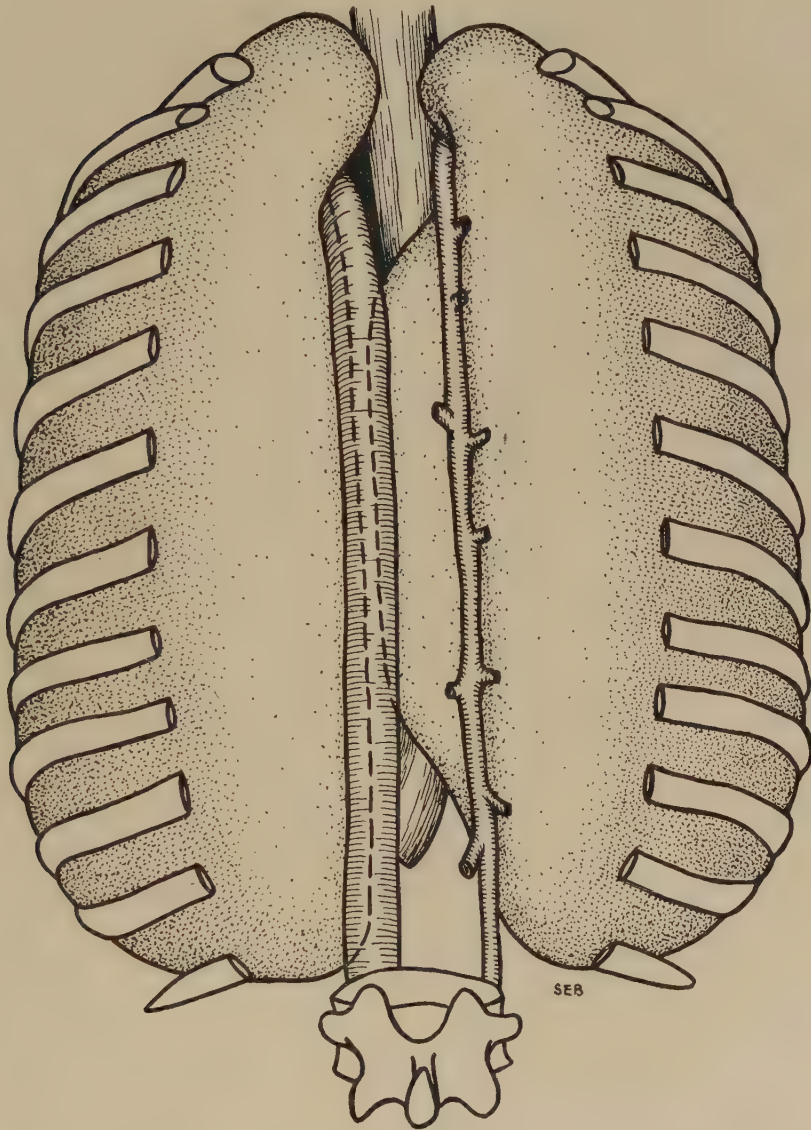


Fig. 6 .Posterior pleural boundaries seen from behind. Pleural cavity has been injected and vertebral column and posterior rib portions have been removed. The figure demonstrates the right pleural cul-de-sac posterior to the esophagus and the approximation of the mediastinal pleurae. Behind the pleural sacs are seen the azygos vein on the right and the aorta on the left. Note also the indentations produced by the arches of the azygos vein and aorta in the upper mediastinum. (Modified from Heiss.)

In figure 2 a the lungs and pleural cavities are represented as being separated posteriorly by a wide mediastinal space in which the esophagus is found directly in front of the spinal column. In contrast to this the corrected diagram (fig. 2 b) depicts the esophagus overlapped posteriorly by the retro-esophageal portions of lung and pleura, and a mesoesophagus is formed by the approximation of right and left pleural septa.

How common is the occurrence of this retro-esophageal pleural recess? Heiss found it in 70% of his cases, and states further that the rest of his cadavers showed irregular posterior pleural boundaries which almost never confirmed the conventional textbook description. On the part of radiologists only one figure is available, viz., that by Stéphan and Kirsch ('36), who observed expansion of the right lung into the mediastinal region beyond the midline in 30% of their cases. According to Bársony and Wald ('36) this lung expansion is especially common in children and in subjects with pronounced dorsal curvature. The upright posture is required in order to visualize on the roentgenogram the maximum medial extent of the lungs; in the supine posture the lungs recede from the medial cul-de-sac.

Whereas anatomists have been concerned mainly with distribution of the pleurae in the arrangement here described, radiologists have focussed most of their attention on the extension of the lungs, thus demonstrating anew how the two procedures supplement each other. But certain discrepancies still await explanation, the main one being the fact that radiologists have observed the maximum medial extent of the lungs in the upper mediastinum, while anatomists have described the most pronounced development of the culs-de-sac in the lower mediastinum. And to make matters still more confusing, pleural shadows can frequently be seen on the roentgenogram lateral to the sides of the spinal column, thus apparently contradicting anatomical observation. These differences can be explained by the fact that portions of the lungs protruding medially in the lower part of the mediastinum are obscured by the shadow of the heart on the roent.

genogram, and that the presence of pleural lines farther laterally corresponding to the most posterior portions of the visceral and/or parietal pleura does not disprove or exclude the presence of pleural recesses more anteriorly behind the esophagus, as demonstrated in figure 4. Some questions must remain open; thus it is impossible to decide how far the lungs penetrate into these recesses and what movements the retro-esophageal portions of the lungs undergo during respiration, since the behavior of the lungs in relation to these culs-de-sac has not been explored sufficiently. As will be pointed out later, the recess under discussion shares this uncertainty with other and better known pleural sinuses. But from the facts at hand it seems necessary to include the retro-esophageal recess in the list of pleural sinuses, where the latter are defined as spaces produced by reflections of parietal pleura and intermittently filled with lung. Mediastino-vertebral recess and mediastino-esophageal recess are the names advanced in the literature for this sinus. Vertebro-esophageal recess seems on the whole a more descriptive term.

B. THE POSTERIOR INFERIOR BOUNDARIES OF LUNGS AND PLEURA

Conventional anatomical teaching

The posterior inferior limits of the pleura are usually defined by a horizontal or medially ascending line which reaches the twelfth thoracic vertebra at a point corresponding to the twelfth costo-vertebral joint.³ According to other descriptions it may come in contact with the spinal column halfway between the head of the twelfth rib and the transverse process of the first lumbar vertebra or even reach the level of the transverse process of the first lumbar vertebra.

Textbook descriptions on the position of the posterior lower lung margins are contradictory and confusing since they often

³ The Morris ('33) diagram (fig. 1075) on the posterior boundaries of lungs and pleura from Rauber-Kopsch was eliminated by these latter authors from their 12th edition ('22), and was replaced by a corrected diagram incorporating the findings of Heiss on the mediastinal pleura and placing the inferior pleural boundaries one vertebra lower.

fail to make clear under what conditions of lung expansion the boundaries have been determined. Also not enough attention is paid to the fact that the limits given are those derived from the cadaver, fresh or preserved as the case may be. In the embalmed cadaver the location of the lower lung boundaries corresponds approximately to the "neutral" position in the living, i.e., a state of quiet breathing (Braus, '24; Cunningham, '37), while in the unpreserved cadaver the diaphragm has reached its highest possible position and the lungs are maximally collapsed.

According to conventional descriptions, based on expiration in the living and often on conditions prevailing in the cadaver, the lower posterior lung margin runs in a horizontal direction, crossing the tenth rib medial to the scapular line. It reaches the spinal column at the upper border of the eleventh rib, i.e., the level of the tenth thoracic spinous process. In the preserved cadaver the inferior lung margin reaches the paravertebral line in the interval between the tenth and eleventh thoracic spinous processes. In inspiration the lungs are said to extend to the level of the spinous process of the eleventh thoracic vertebra. This latter finding is obtained by percussion, which is, besides roentgenography, the only possible means of identifying the respiratory excursions of the lower lung margins. But while change in the percussion note due to inspiration is easily elicited, an exact determination of the lowest extent of the lungs requires much experience and even then is not free from subjective interpretation. Due to varying methods of approach there is no consensus of opinion as to whether the lungs completely fill the costo-diaphragmatic sinuses in deepest inspiration. Some authorities concede it, others deny it. The presence of a glistening mesothelial layer on the inner surface of the pleural reflection makes it certain that movement does occur between the costal and diaphragmatic pleurae down to the lowermost limit of the sinus, otherwise, and by reason of an elementary process peculiar to mesothelial contacts in general, fusion between the two membranes would take place (De Garis, '41). But whether, even

in deepest inspiration, the lungs invade the lowermost portion of the sinus cannot be decided at the present time. Besides the respiratory phase the following additional factors influence the position of the diaphragmatic pleura and of the lower lung margin: (1) age, (2) constitutional factors, (3) posture. (1) The diaphragm and with it the pleural sac and inferior lung margin undergo a general descent during the life span of the individual. The quasi-physiological emphysema of old age leads to a general lowering of the inferior lung borders. (2) The viscera are low in the slender, asthenic thorax, the diaphragm is high and the lungs are relatively short in the hypersthenic individual. (3) Lower lung margin, diaphragmatic pleura, and diaphragm are higher in the supine posture than on standing. With the subject lying on one side, the diaphragm is higher on the prone side than on the other. In the prone posture the diaphragm and consequently the thoracic viscera are displaced upward by pressure from the abdominal viscera.

Roentgenographic findings

What information on the inferior margins of lungs and pleura can the roentgenographic approach furnish? These boundaries have only been investigated by a few authors (Ottonello, '32 b; Bársony and Koppenstein, '33; Peltason and Neumann, '33; Korol, '35) although they can be observed not infrequently on the x-ray film. We found them in approximately 15% of abdominal films taken in our hospital. Their radiographic appearance is usually incidental to study of the abdominal viscera. Consequently they have as a rule been demonstrated with the subject in the prone position, the x-rays being directed through the body in dorso-ventral direction. In this projection we frequently find a line running horizontally or with medial ascent or upward concavity from the lateral thoracic wall toward the spinal column at any level from the upper margin of the twelfth thoracic vertebra to the lower margin of the second lumbar vertebra. Close to the lateral margin of the vertebral column the line turns upward

and can be followed in its vertical course for several vertebrae. Together with the contour of the cupola of the diaphragm cranial to it, this line delimits a translucent space of approximately oval shape in which may be observed lung markings corresponding to the ramifications of the pulmonary artery. The latter can be seen extending down close to the line under discussion, but never beyond it. On bronchograms, which demonstrate the bronchial tree by means of a contrast medium, the lowest extent of the alveolar filling also corresponds in form and position to the line (Korol, '35). The anatomical substrate of this line is the posterior costo-diaphragmatic reflection of the pleura (Peltason and Neumann, '33; Korol, '35). The medial and upward prolongation of the line represents the lowest portion of the posterior mediastinal pleura. The oval space just described corresponds to that part of the lower lobe of the lung which is located behind the dome of the diaphragm.

The question may be raised whether the line under discussion could not be produced by visceral pleura, adjacent to the lower lung margin. While this cannot be definitely excluded, observations by Korol speak against it. This author noticed the line in cases of pneumothorax where the lung is collapsed and the visceral pleura widely separated from the parietal diaphragmatic pleura. But it cannot be denied that the visceral pleura may have an additive effect on the density of the line in the roentgenogram.

The prone position and the inspiratory phase are especially favorable for visualization of the line and space, since the posterior portions of the lung are then maximally aerated and attain their greatest expansion into the costo-diaphragmatic sinus, thus producing conditions of optimal contrast. Yet our own film material which invariably was taken in arrest of quiet breathing in the so-called neutral respiratory position showed the pleural line and the adjacent pulmonary translucency in approximately 15% of all abdominal cases.

The line under discussion is subject to wide variations in form and position. Figures 7 and 8 illustrate these variations

in normal cases from our own material. Each of the lines in figure 7 represents one or more cases in which the pleural line is clearly visible in the manner given. Although the films were all taken in approximately the same respiratory phase, we observe a wide range in location of the line from the upper margin of the twelfth thoracic vertebra to the second lumbar vertebra. In but few cases (six out of forty-six) does the line correspond to the one given in Cunningham ('37) and in

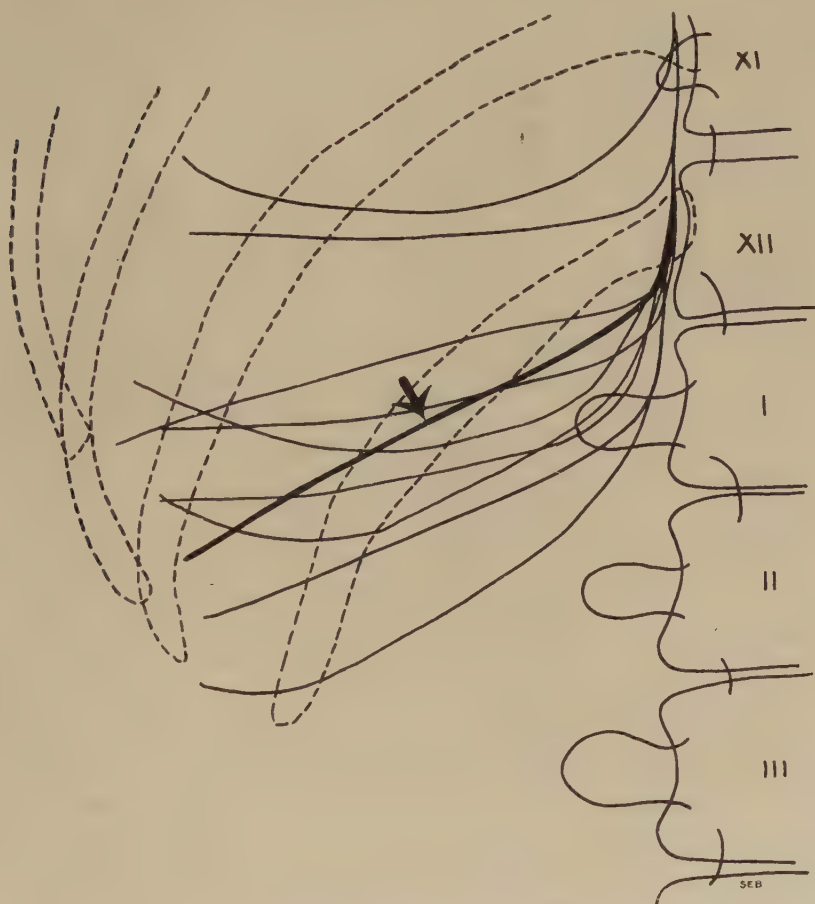


Fig. 7 Figure illustrates variations in the course of the lower pleural line. Arrow indicates the line of pleural reflection as given by Cunningham.

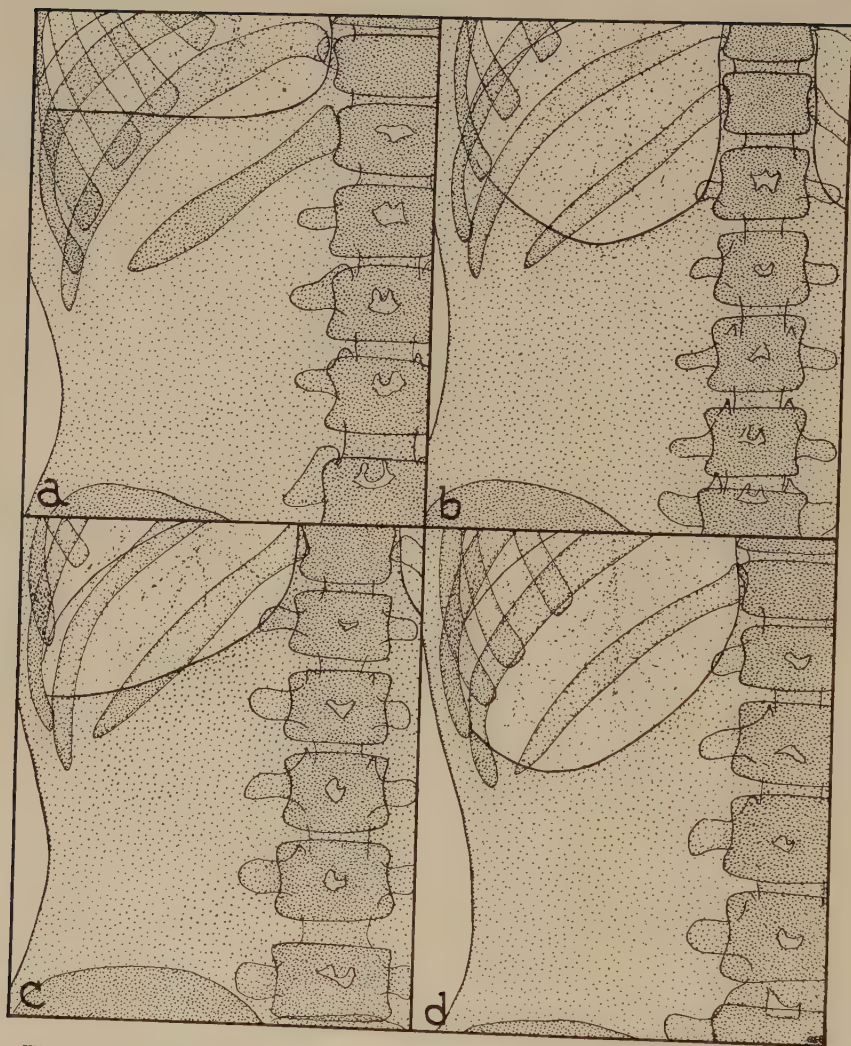


Fig. 8 Diagrams taken from roentgenograms of the right hypochondriac region. Note horizontal course of the pleural line in "a", its medially ascending direction in "c", and its upward concavity in "b" and "d". The level of the line corresponds to the upper margin of the twelfth thoracic vertebra in "a" and to the lower margin of second lumbar vertebra in "d".

three to the pleural margin given in Morris ('33). Especially worthy of note is the upward concavity, which is so common in our material but which has not been mentioned at all in anatomical descriptions. In a few cases the line appears wavy. The pleural line is more common on the right side, but if it is visible on both sides, then its course and appearance are usually identical on the two sides. The position of the line varies with the build of the individual. In the asthenic subject the line reaches its greatest concavity and lowest level (fig. 8 d). In a few cases we were able to demonstrate the pleural line also on films taken in supine position, but the location does not differ essentially from that observed in prone subjects. We have not observed the pleural line on films taken in the upright position.

Discussion

In comparing the results of our radiographic investigations with anatomical presentations of the subject we find that the posterior inferior margins of lungs and pleura in the prone and supine positions reach a lower level than is usually attributed to them in anatomical descriptions. We further notice that the frequently observed upward concavity of lower lung margins and pleura has been overlooked. If we regard the costo-diaphragmatic pleural reflection as the anatomical substrate of the line under discussion which is most probably the case, then the great variability in shape and position of this reflection is well worth noting, as is its lowest possible extent in the neutral respiratory phase down to the level of the second lumbar vertebra. In deep inspiration and in the upright posture the lowermost site of the reflection may be still farther caudad. For practical purposes, e.g., operations on the prone patient, it is necessary to keep well in mind the possibility of the low position of lung and pleura. If, as is less likely, the visceral pleura produces the line in question, then our concept of the relations of lung to pleural recesses in different respiratory phases requires modification. In that

case the lung of the prone subject even in the neutral respiratory position must extend deep into the sinus, perhaps may even fill it completely. In any case, regardless of whether the visceral or parietal part of the pleura produces the pleural line, the lung invades the sinus in horizontal posture to a greater extent than we are accustomed to believe, since lung markings and pulmonary translucency on the roentgenogram can be traced down to a level which is usually taken as typical for the costo-diaphragmatic pleural reflection.

Summary

Results of roentgenographic studies on posterior mediastinal and posterior inferior boundaries of lungs and pleura are not in complete agreement with conventional anatomic teaching. According to anatomic texts the lungs and pleural cavities are separated posteriorly by a wide mediastinal space in which the esophagus is placed directly in front of the spinal column. In contrast to this, roentgenograms taken in the upright posture sometimes depict the presence of a prevertebral space which is occupied by lung and pleura. These findings are in keeping with older continental anatomical presentations which likewise reported the frequent occurrence of a retro-esophageal pleural recess.

The posterior inferior pleural reflection is characterized roentgenographically by a line running horizontally or with medial ascent or upward concavity from the lateral thoracic wall toward the spinal column at any level from the twelfth thoracic to the second lumbar vertebra. This line corresponds to the posterior costo-diaphragmatic reflection of the pleura. A translucency cranial to it is due to inferior lung portions behind the diaphragmatic dome. Anatomic texts usually place the inferior pulmonary and pleural boundaries higher, and disregard the commonly found upward concavity. Reasons for the described discrepancies in the results of radiographic and anatomic approaches are given and the practical importance of the roentgen findings is pointed out.

LITERATURE CITED

- ARENDE, J. 1933 Zur Pathologie des Mediastinums. Mediastinale Randstreifen, Mediastinitis und Mediastinalempysem. Fortschr. a. d. Geb. d. Roentgenstr., Bd. 48, S. 1-13.
- BÁRSONY, TH., AND E. KOPPENSTEIN 1933 Das sagittale Roentgenbild des rechten hinteren Pleurasinus und pathologische Veränderungen desselben. Roentgenpraxis, Bd. 5, S. 170-173.
- BÁRSONY, TH., AND BÉLA WALD 1936 Das Roentgenbild der oberen hinteren schwachen Stelle des Mediastinums. Der praevertebrale, retrooesophageale Lungenteil. Roentgenpraxis, Bd. 8, S. 88-95.
- BRAUS, H. 1924 Anatomie des Menschen. Zweiter Band: Eingeweide. Berlin.
- CUNNINGHAM, D. J. 1937 Textbook of Anatomy. 7th ed. New York.
- DANELIUS, G. 1929 Experimentelles ueber den Verlauf der oberen Lungengrenze im Roentgenbilde. Fortschr. a. d. Geb. d. Roentgenstr., Bd. 40, S. 249-261.
- 1931 Roentgenologie der oberen medialen Lungenabschnitte. Fortschr. a. d. Geb. d. Roentgenstr., Bd. 44, S. 626-634.
- 1933 Zur Frage der "pleuritischen Mediastinalstreifen." Fortschr. a. d. Geb. d. Roentgenstr., Bd. 47, S. 271-275.
- DE GARIS, C. F. 1941 The anatomy of the gastrointestinal tract. In S. A. Portis: Diseases of the digestive system. Philadelphia.
- FANCONI, G. 1931 Zur Diagnose der Pleuritis mediastinalis fibrosa im Kindesalter. Die "pleuritischen" Mediastinalstreifen. Roentgenpraxis, Bd. 3, S. 49-51.
- GRANT, J. C. B. 1940 A method of anatomy. 2nd ed. Baltimore.
- HEISS, R. 1919 Ueber die hinteren Pleuragrenzen. Arch. f. Anat. u. Physiol., Anat. Abt., S. 130-136.
- KOROL, E. 1935 The lower lung line. Am. J. Roentg. & Radium Ther., vol. 34, pp. 740-743.
- MAIER, H. C. 1940 Roentgen aspects of the upper retro-oesophageal pulmonary borders. Am. J. Roentg. & Radium Ther., vol. 43, pp. 168-172.
- MERKEL, F. 1902 Handbuch der Anatomie des Menschen. Herausgegeben von K. v. Bardeleben. Darmsystem. Erste Abteilung: Atmungsorgane. Jena.
- MORRIS 1933 Human Anatomy. 9th ed. Philadelphia.
- OTTONELLO, P. 1932 a Bemerkungen zur normalen Roentgenanatomie des Thorax. Fortschr. a. d. Geb. d. Roentgenstr., Bd. 45, S. 677-687.
- 1932 b La rappresentazione radiografica dello sfondato pleurico posteriore. Radiol. med., vol. 19, pp. 469-476.
- PELTASON, F., AND W. NEUMANN 1933 Die Roentgendarstellung der unteren Pleuragrenze. Fortschr. a. d. Geb. d. Roentgenstr., Bd. 47, S. 520-529.
- POIRIER, P., AND A. CHARPY 1901 Traité d'anatomie humaine. Tome Quatrième. Paris.
- PRATJE, A. 1924 Zur Topographie des Mediastinums am Lebenden. Anat. Anzeiger, Bd. 58, Erg. Heft, S. 89-99.
- RAUBER-KOPSCH 1922 Lehrbuch und Atlas der Anatomie des Menschen. Bd. 4. 12th ed. Leipzig.
- SPALTEHOLZ, W. Handatlas of human anatomy. Vol. 3, 7th ed. Philadelphia.

- STÉPHANI, J., AND R. KIRSCH 1936 Étude radiographique de l'expansion interne du poumon droit. Condition nécessaire pour qu'elle se réalise et que son image radiographique apparaisse. *Press. méd.*, t. 44, pp. 1585-1586.
- TESTUT, L. 1905 *Traité d'anatomie humaine*. Tome troisième. Paris.
- WECHSLER, Z. 1931 Die "pleuritischen" Mediastinalstreifen im Kindesalter in ihrer klinischen Bedeutung. *Fortschr. a. d. Geb. d. Roentgenstr.*, Bd. 44, S. 81-86.
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EXPERIMENTS ON THE SOURCE OF OVARIAN ANDROGEN IN THE MOUSE¹

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TWO PLATES (TWENTY-TWO FIGURES)

It has been demonstrated that after a sufficient period of residence in castrated males, transplanted ovaries will maintain in a normal condition the male accessory reproductive glands of guinea pigs (Lipschütz, '32), mice (De Jongh and Korteweg, '35; Hill and Gardner, '36; Hill, '37 a and b; Hill and Strong, '38) and rats (Deanesly, '38). It has further been shown that masculinization of the female guinea pig results from the endocrine imbalances caused by the removal of one ovary and part of the other (Lipschütz, '33, '37) and by irradiation of the ovaries with x-rays (Steinach and Kun, '31). The female guinea pig is also readily masculinized by injections of certain gonadotropic preparations (Steinach and Kun, '31), extracts of hypophyses, and urine of pregnant women (Guyénot, et al. '32, '33, '34, '36; Papanicolaou and Falk, '34), and in the interspecific cross *Cavia aperea* X *Cavia cobaya* (Guyénot, et al. '35). Masculinization has also been induced in young female rats by the injection of gonadotropic extracts (Bradbury and Gaensbauer, '39; Greene and Burrill, '39 a, b). Apparently, however, these extracts are effective only if given during the first 30 days of life, suggest-

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ing an endocrine imbalance rather than direct stimulation of the ovary to produce male hormone.

In the rat luteinized cells of the theca interna are probably involved in the production of ovarian androgen since demonstrable production of androgen by ovarian grafts occurs only in the presence of extensive thecal luteinization (Deanesly, '38). In other species no particular ovarian element has been clearly associated with the androgen production. The presence of large numbers of "luteal-like" cells in androgen-producing ovarian grafts in the ears of castrated male mice (Hill, '37 a) suggests these cells as a possible source of the androgen, but their involvement is reported to be questionable (Hill, '41). Whether luteinized cells of the theca interna are concerned is more difficult to ascertain in the mouse than in the rat. In the latter species luteinization is limited to the theca in ovaries transplanted to males (Goodman, '34; Pfeiffer, '36; Deanesly, '38) while in the mouse either no luteinization (Gardner, '35) or both thecal and granulosa luteinization is found in ovaries transplanted to males. Similarly, luteinization of both tissues occurs in female mice with masculinized hypophyses (Pfeiffer, '39).

Two procedures suggest themselves as potential means of determining the site of production of ovarian androgen in the mouse: (1) If massive treatment with any of several gonadotropic preparations should increase or hasten the production of androgen in appropriate test animals, this function might be accompanied by some particular ovarian histology which would indicate the source of the androgen. (2) The involvement of "luteal-like" cells could be investigated by experimentally increasing the number of these elements in the ovary and determining whether there is a corresponding increase in androgen production. Since pregnant mare serum provokes luteinization of most of the elements of the ovary, this substance affords a means of inducing the formation of "luteal-like" cells. Moreover, Evans, et al. ('37) have indicated that administration of P. M. S. leads to the production of androgen by the rat ovary.

Ovarian grafts in the ears of castrated males constitute the most satisfactory arrangement for detecting ovarian production of androgen, inasmuch as fluctuations in the amount of androgen can be followed by the effects on the seminal vesicles and prostate glands. Unfortunately, under these conditions the grafting factor is always present as a potential source of confusion in interpreting histological details in the ovary. In the intact female, on the other hand, this factor is not present; and the production of androgen, as well as of estrogen and progestin, may be followed by study of the endometrium which exhibits an apparently specific histological response to each of these hormones (Hooker, '40). Estrogen evokes edema and heightening of the epithelium, progesterone causes the stromal nuclei to become vesicular, while testosterone causes the tunica propria to become more fibrous. Moreover, the length of the survival period following adrenalectomy provides a criterion for determining whether heavily luteinized ovaries are producing progesterone in large amounts (Pfeiffer and Hooker, '40). For these reasons intact females were used as well as castrated males with ovarian grafts in testing the effects of all of the gonadotropins used in the present experiments.

MATERIALS AND METHODS

Since preliminary experiments showed that the interstitial cells in the ovaries of mice of the A strain of Strong ('36) increase greatly in number and undergo alteration under the action of several gonadotropins, this strain was selected for these experiments. Fifty-nine males were castrated and, at the same operation, ovaries from litter-mates were transplanted to the ears by the method of Hill and Gardner ('36). Beginning at operation, daily subcutaneous injection of several gonadotropic substances was made as follows: Twenty-nine animals received pregnant mare serum², the daily dose for

² Pregnant mare serum (Gonadin), which for the sake of brevity will be referred to as P. M. S., was kindly furnished by the Cutter Laboratories through the courtesy of Drs. D. H. Wonder and C. Parham. The dosage is expressed in terms of the Cole-Saunders rat unit, which is biologically equivalent to two international units.

nineteen being 20 R. U. and for each of two groups of 5 being 10 R. U. and 5 R. U. respectively. Five animals were treated with 20 R. U. of a pregnancy urine extract ³ and five with 2 mg. of whole anterior lobe powder ⁴. Five mice were given a preparation containing 2.5 R. U. of follicle stimulating hormone ⁵ in each daily dose, and five received 2 mg. of a luteinizing hormone preparation ⁶. The doses of whole anterior lobe powder and of the luteinizing hormone were the maximum amounts tolerated without ulceration at the site of injection. The amount of F. S. H. administered was limited by the concentration of the preparation available.

In all cases female litter-mates of the operated animals received the same treatment to permit a comparison of the response of the ovary in situ with that of the grafted ovary. Ten castrated males bearing ovarian grafts in the ears were kept untreated to provide a basis for evaluating the effects of the various treatments. With a few exceptions the animals were between 30 and 40 days of age at the beginning of the experiment. All of the animals were maintained on a diet of Purina Fox Chow. Water was available at all times.

The seminal vesicles and prostate glands were used as indicators of androgen activity in the host animals, and their state of development was followed by weekly laparotomies. If their gross appearance indicated stimulation, biopsies of the seminal vesicles were taken. The condition of the ovarian graft was followed by periodic examination, aided by projecting a strong light through the pinna. In the females the histology of the endometrium was used as an indicator of the identity and relative amounts of the various hormones

³ Pregnancy urine extract, which will be referred to as P. U., was kindly furnished by E. R. Squibb and Sons through the courtesy of Drs. J. A. Morrell and J. F. Anderson.

⁴ The whole anterior lobe powder (A. P.) used was Wilson's acetone dried whole sheep pituitary powder.

⁵ The follicle stimulating hormone, Prephysin (F. S. H.), was obtained from Chappel Laboratories.

⁶ The luteinizing hormone (L. H.) was prepared from the whole sheep pituitary powder according to the method of Fevold.

released. At autopsy the grafts, ovaries, uteri, adrenals, seminal vesicles and prostates were preserved in Bouin's fluid, sectioned at 10 μ and stained with Ehrlich's hematoxylin and triosin.

GENERAL OBSERVATIONS

Pregnant mare serum treatment. While castrated males bearing ovarian grafts and maintained without treatment failed to exhibit evidence of androgenic action for at least 130 days, similar animals receiving 20 R. U. daily of pregnant mare serum showed distinct stimulation of the seminal vesicles after 35 days (fig. 3). By 60 days the seminal vesicles were as fully developed as in normal intact males (fig. 4), indicating that the ovarian graft was sufficiently androgenic to result in full development of the male accessory sex organs. After 60 days the grafts were removed from certain of the treated animals and one half of each was fixed for histological study (fig. 2). The remaining portion of the graft was implanted into other males which were immediately castrated. In their new hosts the grafts progressively diminished in size despite the daily administration of 20 R. U. of P. M. S. Production of male hormone in amounts that could be detected by an effect upon the seminal vesicles ceased, and the graft fibrosed, indicating that the cells responsible for the production of the androgenic substance could not be transplanted.

When 10 R. U. of P. M. S. were injected daily into castrated males bearing ovarian grafts, only slight stimulation of the seminal vesicles was detected after 45 days, and this in only two of five mice. Even 60 days of this treatment failed to result in stimulation as great as that found after 35 days in the animals receiving daily injections of 20 R. U. The seminal vesicles were maintained in this slightly stimulated condition until treatment had continued for 90 to 100 days, when they began to atrophy slowly to the castrate condition simultaneously with regression of the grafts. Administration of 5 R. U. daily did not result in a definite increase in the size of the seminal vesicles until after 60 to 90 days of treatment.

Stimulation then progressed until some vesicular fluid was present, but never equaled that obtained with 10 R. U.

The ovaries of intact females receiving either 20 or 10 R. U. of P. M. S. were five or six times normal size after 8 months of treatment. At about 1 year of age, when the animals developed mammary tumors and were autopsied, the ovaries were still approximately twice normal size. The response was practically the same with 5 R. U.

Pregnancy urine preparation. When 20 R. U. of P. U. were injected daily, some of the largest grafts observed in the entire study developed. Within 15 to 25 days the seminal vesicles were larger than those of untreated castrates with ovarian grafts, and appeared to contain a small amount of clear fluid. Histologically, however, the epithelium of the seminal vesicles showed little or no stimulation (fig. 7); but an increased thickness of the stroma indicated the action of estrogen. There was no distinct evidence at any time of male hormone production, while the estrogen effects became more marked as treatment continued until about 100 days, after which they diminished somewhat, and the animals were autopsied.

As indicated by uterine and vaginal morphology the ovaries of intact females receiving 20 R. U. of P. U. daily were strongly stimulated at first but by 35 days were little larger than normal. At 100 days, when the animals were autopsied, the ovaries were smaller than those of untreated control females, but were still producing sufficient estrogen to elicit uterine and vaginal responses.

Whole anterior pituitary powder (sheep). The ovarian grafts in the animals receiving 2 mg. of this preparation daily increased in size and remained active for 30 to 50 days, after which time they regressed. The seminal vesicles remained castrate in type (fig. 8). The ovaries of the females which received this treatment became refractory and were much smaller than those of normal animals after 45 to 60 days of treatment.

Follicle stimulating and luteinizing hormones. Since all of the gonadotropic preparations used in the preceding experiments contained both F. S. H. and L. H. in different amounts, it was possible that one of these substances might have been the principle in the P. M. S. responsible for the production of male hormone by the ovaries, but not present in sufficient quantity in the other two preparations to be effective in this respect. Accordingly, the effects of F. S. H. and L. H. were tested separately, using the most purified preparations of these principles available.

Injections of 2.5 R. U. daily of F. S. H. did not evoke the production of male hormone (fig. 11) by the ovarian grafts. The grafts as well as the ovaries of intact females receiving this treatment became refractory within a month, that is, before release of male hormone could be expected on the basis of the time relations with P. M. S.

Administration of 2 mg. of L. H. daily did not result in appreciable growth of the graft or in the production of evident amounts of male hormone (fig. 12), nor did the ovaries of intact females receiving this treatment show a macroscopically visible change at any time, except for a possible decrease in size. The possibility has not been eliminated, however, that higher doses of F. S. H. and of L. H. might be effective.

Androgenic action of progesterone. It has been tacitly assumed above, for ease of description, that the androgenic action of the ovarian grafts was due to their secretion of a male hormone. The possibility existed, however, that this androgenic action was exerted by progestin since Greene and co-workers ('39) have described androgenic effects of administered progesterone, and since the grafts and ovaries were heavily luteinized following treatment with P. M. S. Using survival of adrenalectomized mice of the A strain (Pfeiffer and Hooker, '40) as a criterion, it was found that the amount of progesterone produced by the ovaries at their most active period (21 to 35 days of treatment) in response to P. M. S. was equivalent to daily injected amounts of between 0.5 and 1.0 mg. To determine whether this amount of progesterone

is andromimetic, seven males were castrated and two of these were injected with 0.6 mg. and three with 1.0 mg. of progesterone daily, while the remaining two were left untreated. No difference was observed in the rate of regression of the accessory glands of reproduction in the treated and control animals, showing that the androgenic effects observed in the grafted animals receiving P. M. S. could not have been due to progesterone.

HISTOLOGICAL STUDY

Since of the several gonadotropins which stimulated growth of the grafted ovaries only P. M. S. induced appreciable production of male hormone, a comparative histological study of the grafts and of ovaries in intact females was made with reference to their responses to the various gonadotropins. The histology of the grafts and ovaries agreed very closely (compare figs. 1 and 2 with 16, 17, 18 and 19), with the exception that the latter showed more obvious and uniform gradations in their response because of their freedom from the effects of grafting. For this reason it was more convenient to use the ovaries of intact females in determining the sequence of histological events leading up to any given stage in the response to treatment.

Ovaries of control mice of the A strain

The ovaries of mice of the A strain are sufficiently different histologically from those of the common laboratory mouse to necessitate some description. It is characteristic of this strain that very few old corpora lutea are present in any stage of the cycle, although oogenesis appears to be entirely normal. Follicles of all stages of development are present; and apparently the usual number ovulate, following which the ova can be found in the sacculated portion of the tube, as would be expected. During pro-estrus large preovulatory follicles may be seen to be on the point of rupturing. Ruptured follicles showing beginning luteinization are found within 12 hours after ovulation (fig. 13), and enlarging basophilic cells

of the forming corpora lutea are present for 24 hours or slightly longer (fig. 14). The corpus luteum does not appear to develop beyond this stage but, instead, rapidly loses its basophilic character, and ingrowths of connective tissue from the theca externa break it up into small masses of cells which are added to the stroma. These cellular masses may form medullary cords, or cords extending centrally from the periphery, or they may be present as small irregular nests throughout the ovary. The number of cells included in these masses varies greatly, and each mass is separated from others by a narrow strip of dense stroma, which in certain cases almost amounts to a surrounding membrane. By the time the process of dispersion is completed most of the cells in the masses are agranular and have pycnotic nuclei, although in old females a few large cells with eosinophilic granules are also present. The cells in these masses are the characteristic ovarian interstitial cells of the A strain of mice. A few structures distinguishable as corpora lutea may be found between this time and the next ovulation, but they are never as numerous as the ova liberated at the last ovulation.

Stimulation with pregnant mare serum

The changes in the ovary resulting from administration of 20 R. U. of P. M. S. daily were followed in a group of female mice by sacrificing one animal each day for histological study during the first week of treatment and one animal every third or fourth day thereafter until the injections had continued 75 days. A summary of these changes and of the accompanying hormone production is given in table 1. During the first 3 days of treatment the ovarian response consisted of marked follicular growth, supernumerary ovulation, and normal luteinization of the ruptured follicles, without any apparent increase in the number of primary follicles or interstitial cells. By the end of the third day the number of well formed, medium-sized follicles was about the same as that found in normal animals, but there was an increase in the number of large pre-ovulatory follicles. At this stage of treatment most of the

TABLE 1

Summary of the chief histological features of, and the hormones secreted by, the ovaries of intact female mice of the A strain following the administration of 20 R. U. of P. M. S. daily.

DAYS OF TREATMENT	HISTOLOGICAL CHANGES IN THE OVARY			UTERINE RESPONSES		
	Number of follicles	Amount of active luteal tissue	Interstitial cells	Estrogen	Progesterin	Androgen
3	Increased	Normal	Normal	++	+	—
7	Increased	Thecal luteinization	Eosinophilic cells	+++	++	—
10	Increased	Increased	Some eosinophilic cells very slight vacuolization	++++	++	+
14	Increased	Increased	Increased number of eosinophilic granular cells	+++	++	+ ?
17	Approx. normal	Appreciably increased	Increased number of eosinophilic granular cells	+++	+++	+
21	Approx. normal	Appreciably increased	Increased number of eosinophilic granular cells	++	+++	?
24	Approx. normal	Extreme	A few vacuolated eosinophilic granular cells	+	++++	?
28	Approx. normal	Extreme	A few vacuolated eosinophilic granular cells	+	++++	+
31	Approx. normal	Extreme	Appreciable numbers of vacuolated eosinophilic cells	+	++++	++
35-38	Approx. normal	Increased	Increased number of vacuolated eosinophilic granular cells	+	+++	+++
42-45	Approx. normal	Increased	Increased number of vacuolated eosinophilic granular cells	+	++	++++
49-66	Approx. normal	About normal	Both unvacuolated and vacuolated eosinophilic granular cells	+	+	++++
70-75	Approx. normal	About normal	Both unvacuolated and vacuolated eosinophilic granular cells	+	+	++++

+ A detectable response to the hormone.

++ Definite response.

+++ Marked response.

++++ Maximum response.

follicles ovulated normally, and well delineated corpora lutea formed. However, some of the pre-ovulatory follicles showed marked thecal luteinization (fig. 15). This thickening of the theca interna was possibly responsible for the frequent occurrence at later stages of luteinization of unovulated follicles, since it may have constituted a mechanical obstacle to their rupture. It is probable that the normal number of primary follicles was present at this time; but the increased size of the ovary, resulting principally from the increase in the number of large follicles and well developed corpora lutea, caused the primary follicles to be more dispersed and consequently to appear less numerous. The cords of agranular interstitial cells between the corpora lutea and follicles did not appear to be affected by the treatment at this time. These interstitial cells, by their faintly acidophilic character, contrasted sharply with the more basophilic corpora lutea. No increase in mitotic activity of the germinal epithelium was observed. By the seventh day luteinization of the theca interna occurred more frequently and the interstitial cells were more numerous. The genital organs showed an increasing hyperemia characterized by dilated sinusoids in the newly formed corpora lutea and distention of the vessels in the medulla of the ovary and in the uterus. The endometrial stroma was thickened and distinctly edematous, and the endometrial epithelium was composed of relatively tall, apparently active, granular cells, indicating the action of estrogen. Some of the stroma cells were enlarged and contained the large ellipsoidal nuclei seen after progesterone treatment.

By the tenth day corpora lutea were even more numerous, a few having formed without ovulation. In certain areas the older corpora lutea were being broken up into smaller masses of cells by the ingrowth of fibrous strands from the theca externa, and these cell masses were being added to the interstitial tissue of the ovary. The history of these cells was similar to that of the interstitial cells of normal mice of the A strain except that they remained in the corpora lutea as active luteal cells for a longer period. Like the normal inter-

stitial cells, they were predominantly agranular and had pycnotic nuclei. On the other hand, they contained more cytoplasm, probably due to their having reached a more advanced stage of development in the corpora lutea. The transformation from the luteal to the agranular type of cell took place within the older corpora lutea. These interstitial cells are identical in appearance with the "luteal-like" cells described by a number of authors as occurring in the ovary under various experimental conditions. It was primarily the formation and persistence of this type of interstitial tissue which accounted for the greater size of the ovary at this time. Small isolated clumps of these cells were also present in the medulla of the ovary between the distended blood vessels, but they could not be traced to any recently organized corpora lutea and might have been derived from pre-existing interstitial cells of this region.

A few of the cells in the old corpora lutea and in the cell masses derived from them were not of the agranular type but, instead, contained clumps of vividly eosinophilic cytoplasmic granules, and had nuclei which were either round or ellipsoidal in shape with darkly staining, dispersed chromatin. These cells are not normally found in the ovary, and in animals receiving P. M. S. made their first appearance at this stage of treatment. They had the characteristics of secretory cells and were interpreted, on the basis of conditions observed at later stages of treatment, to be at this time in a pre-secretory or beginning secretory phase. At this time, also, the tunica propria of the uterus began to be slightly fibrous, the first indication of the action of androgen. Thus, the appearance of a male hormone response showed a close temporal relationship to the appearance of the eosinophilic granular cells. Production of estrogen, as evidenced by the character of the uterine epithelium and by the endometrial edema, continued at about the same level as in earlier stages. The condition of the stromal nuclei indicated that progesterone was present in larger amounts than before, doubtless a result of the increase in the number of corpora lutea.

The break-up of the old corpora lutea into cell masses was even more prominent after 2 weeks of treatment, and these cell masses comprised a proportionately greater part of the ovary. By this time the ovary had become more vascular, many of the large follicles were hemorrhagic, and the recently formed corpora lutea showed great vascular congestion, although their cells were normal and appeared to be active. That the cells of the hemorrhagic corpora lutea were functional was evidenced by the fact that more of the uterine stromal nuclei were vesicular than at preceding stages, indicating a further increase in the production of progesterone. The endometrium continued to show a good estrogen response even though the edema was somewhat less marked than previously. The uterine stroma was only slightly fibrous, indicating that male hormone production was still very slight.

The amount of basophilic luteal tissue reached its maximum at 21 days, the increase being partially due to luteinization without ovulation of many follicles. However, ovulation had also continued to occur as evidenced by the presence of ova in the tubes at 21 days. Precocious luteinization had materially decreased the number of large follicles, and all of those present were hemorrhagic, as were also the newly formed corpora lutea which by this time contained greatly dilated sinusoids. The uterus at 21 days exhibited vascular congestion, a low but proliferating endometrial epithelium, and a less fibrous stroma, all of which suggested a diminished production of both estrogen and androgen; while the presence of greater numbers of vesicular nuclei in the stroma of the uterus than seen earlier indicated that progesterone production had further increased.

Throughout the entire period of treatment the ovary showed the various responses to P. M. S. described above, but the different tissues assumed predominance at different times. Thus, between the twenty-first and thirty-fifth days the ratio of normal basophilic luteal cells to altered cells of the type found in the old corpora lutea and in the interstitial cell masses shifted toward the latter. Most of the altered cells had by this

time assumed a more active appearance, the cytoplasm containing eosinophilic granules and the nuclei being vesicular. These eosinophilic granular cells, however, differed from those first seen around the tenth day of treatment in that the granules were smaller and more dispersed and the cytoplasm contained many very small vacuoles. This type of eosinophilic granular cell apparently represented a later secretory condition of the cell containing deeply staining clumps of granules seen on the tenth day. The altered cells which had lost their identity as old corpora lutea by the ingrowth of connective tissue fibers from the theca externa were at this time being arranged into irregular cord-like masses (figs. 16 and 19). This arrangement was presumably caused by internal pressure in the ovary resulting from the rapid addition to the stroma of large numbers of cells of the old corpora lutea and from the continued formation of new follicles and corpora lutea. The number of vascular channels in the stroma of the ovaries had increased, perhaps partially as a result of inclusion of channels which had been present in the old corpora lutea. The endometrium at 35 days had a relatively low epithelium, and the stromal cells continued to show a good progesterone response. The tunica propria was appreciably more fibrous (fig. 21), indicating an increase in the production of androgen. Thus, male hormone production by the ovaries of intact females being treated with P. M. S. closely paralleled that by ovarian grafts in castrated males receiving the same treatment, since at 35 days the state of the seminal vesicles of these males indicated that androgen production was well under way.

Between the thirty-fifth and fortieth days of treatment the process of active formation of corpora lutea became stabilized at about the same rate as in the ovaries of untreated animals. Following this period the principal response of the ovary was a further accumulation in the stroma of the altered cells derived from the old corpora lutea. By the fortieth day the uterine epithelium had become still lower, granules had almost disappeared from the cytoplasm, and mitoses were very rare. The progesterone response was variable, being fairly well

marked in some areas and almost absent in others. The fibrous condition of the tunica propria had continued to increase, and there was no appreciable edema.

After 45 days it became relatively more difficult to distinguish the cells of the old corpora lutea from the altered cells in the stroma because the latter cells now tended to be crowded together into large masses which had an organization resembling that of the old corpora lutea. The old corpora lutea could, however, be identified by the presence of a definite theca externa. The altered cells in both the old corpora lutea and in the stroma remained eosinophilic, although by 45 days the vacuolization of the cytoplasm had become more pronounced and many of the nuclei had irregular outlines. The new corpora lutea, which by this time occupied a relatively small portion of the ovary, could be readily identified by their basophilism. The vascular congestion of the ovary persisted (fig. 18), and in the latest stages studied dilated vessels in the medulla separated many of the masses of altered cells from each other and from the old corpora lutea.

Even though the luteinization of pre-ovulatory follicles had continued, ova were still found in the tubes after 60 days of treatment. By this time much of the germinal epithelium had disappeared, being present only superficial to the tissue (fig. 22) wedged into the spaces between the corpora lutea or masses of altered cells at the periphery of the ovary. A few primary oocytes remained in these nests of cells, and a few growing follicles were present in the deeper portions of the ovary. Only slight evidence of estrogen stimulation was found in the endometrium, while the paucity of enlarged uterine stromal cells indicated a very low production of progesterone. The continued production of male hormone by the ovary was evidenced by the condition of the tunica propria which, although relatively thin, was quite fibrous. From this time until the termination of treatment at 75 days conditions in the ovary and uterus remained essentially unchanged. Some of the eosinophilic granular cells had, however, apparently developed into a larger size before vacuolation and dispersal

of the granules had begun since a few unusually large cells of both the vacuolated and the unvacuolated types (fig. 20) were present during the later stages of treatment.

Treatment with other gonadotropic hormones

The series of ovaries obtained for histological study from intact females treated with F.S.H., L.H., A.P., or P.U. was less closely graduated than the series of ovaries from females treated with P.M.S. However, when supplemented by ovarian grafts in castrated males each series was adequate to show the important changes in the ovary and to indicate the hormones released.

Follicle stimulating hormone. The first response of the ovary to F.S.H. was an increase in the number and rate of development of follicles. Ovulation occurred normally and corpora lutea formed, although at a much slower rate than following P.M.S. stimulation. The corpora lutea were neither very large nor, as judged by the morphology of the endometrium, very active. This condition, together with the fact that all of the follicles luteinized, presumably due to release of pituitary L.H., had the effect that the ovarian mass increased relatively little. The old corpora lutea were broken up by the same process as occurs following treatment with P.M.S.; but the cells promptly became somewhat spindle-shaped, agranular, and chromophobic, and their nuclei became pycnotic, causing them to resemble typical stroma cells rather than interstitial cells. As the treatment continued follicular growth diminished, and the ovaries became fibrous. From 60 to 90 days after the beginning of treatment the ovaries were only one-third to one-half the size of normal ovaries. Only a very few follicles or corpora lutea in any stage of development were present, and the ovaries were comprised mainly of the cells with pycnotic nuclei imbedded in a dense stroma (fig. 9), a condition indicating almost complete physiological hypophysectomy.

The condition of the endometrium indicated that some estrogen was released during the early phases of treatment

with F. S. H. and that none was produced after the ovary became refractory. There was little, if any, evidence of action of progesterone on the stromal cells of the uterus and no evidence of male hormone production.

Luteinizing hormone. The follicles present at the beginning of L. H. treatment luteinized, but only a few new follicles formed, presumably under the influence of the animal's own pituitary. The corpora lutea did not persist as such but, as after F. S. H. treatment, broke up relatively early into small cellular masses which were added to the stroma. The cells in these masses did not, however, transform into stroma cells nor did their nuclei become pycnotic as in the animals receiving F. S. H.; instead, these masses persisted as organized units (fig. 10). Except that in the L. H. treated animals the cells did not develop into vacuolated, actively secreting cells, this picture resembled that in P. M. S. treated animals inasmuch as the cells were maintained unchanged in the stroma. At 90 days the ovaries of L. H. treated animals were even smaller than those obtained by treatment with F. S. H. due to the low degree of follicular development and the consequent paucity of corpora lutea and cell masses derived from the corpora lutea. The morphology of the uterus gave no evidence that male hormone was produced, and indicated that the female sex hormones were present in smaller amounts than in the normal cycle.

Whole anterior pituitary powder (sheep). At first good follicular and luteal stimulation followed treatment with A. P., but the corpora lutea remained intact and basophilic only a short time and then broke up as in the animals receiving the other gonadotropins. By 35 days the ovary showed signs of refractoriness, although a few follicles and corpora lutea could be found throughout the entire period of treatment. At 90 days, when the last animal was autopsied, the ovaries were smaller than after either F. S. H. or L. H. stimulation. The organization of the cell masses in the stroma resembled that obtained upon treatment with P. M. S. and L. H. except that the number of cells in each mass was usually smaller. The

nuclei of the cells in the masses were somewhat pycnotic, but the volume of cytoplasm was relatively large. Thus, these cells closely resembled the agranular cells found during the early stages of P. M. S. treatment. Before the ovary became refractory the condition of the uterus indicated the presence of appreciable amounts of estrogen and some progesterone, but no male hormone.

Pregnancy urine hormones. Follicular development was readily initiated by administration of 20 R. U. of P. U. daily, but the predominating response, even during the early stages, was luteinization. The condition of the ovaries was similar to that seen after administration of P. M. S. in that the cells of the theca interna showed some enlargement and many corpora lutea formed. However, this response was somewhat less marked than after P. M. S. treatment, although the doses were comparable in assay units. The total quantity of luteal tissue never became as great as after P. M. S. treatment due, at least partially, to the fact that the ovary soon became refractory. At 35 days, there were relatively few maturing follicles, although small and primary follicles were present in apparently normal numbers. Most of the corpora lutea had broken up into cords and masses of cells containing a larger amount of cytoplasm than observed after treatment with F. S. H. and L. H., but not nearly so much as that observed following P. M. S. treatment (fig. 5). The apparently extreme paucity of granules in the cytoplasm of most of the cells was probably due to the greater cytoplasmic volume as well as to the actual loss of granules. Vacuolation of the cytoplasm did not occur. A few of the cells retained some granules and were quite acidophilic. A small number of hemorrhagic follicles was present, and there were also a few clumps of large epithelioid cells with a yellow to green granular cytoplasm. These cells were thought to be macrophages containing hemosiderin resulting from the breakdown of old hemorrhagic areas. Such cells are characteristically present in senile ovaries, testes, and adrenals of the mouse. At 90 days the ovary had become quite refractory and resembled the

senile condition. There were still many cords and small masses of cells of the inactive type described at 35 days, but their nuclei had become more pycnotic (fig. 6). About the same number of large "yellow-green" cells were present, although they were scattered throughout the stroma instead of being in clumps. Except for the infrequent presence of a follicle or corpus luteum, there was no ovarian evidence of active hormone production. Estrogen was present during the early period of treatment, as evidenced by the condition of the endometrium, while changes in the uterine stromal cells indicated that progesterone was present, although its production began slightly later than that of estrogen. Both estrogen and progesterone were in evidence beyond 35 days, but their production had apparently almost completely ceased by 90 days. No evidence of male hormone production was detected in the uterus at any time.

DISCUSSION

The most striking histologic feature by which the ovaries of mice treated with pregnant mare serum and producing androgens differed from the ovaries of mice receiving other gonadotropic preparations and not producing androgen was the presence of large masses of vacuolated, eosinophilic, granular cells. Since these cells were vacuolated in only the ovaries producing androgen, since vacuolation appeared simultaneously with the onset of production of androgen, and since vacuolation of these cells was greatest when the production of androgen was greatest, it seems very probable that these cells are the source of the androgen. The fact that unvacuolated, eosinophilic, granular cells are present both before and after the production of androgen (Pfeiffer and Hooker, '42) and are also present in animals receiving luteinizing hormone, either as a purified preparation or in whole anterior lobe powder, suggests that the development of the vacuolated cells is controlled by two factors, the first being responsible for the formation of the eosinophilic granular cells and the second for the vacuolation of these cells. In the presence of both

factors, as would be assumed to be the case in the animals treated with P. M. S., eosinophilic granular cells would form under the influence of one factor and become vacuolated under the stimulus of the other. If only the first factor is active, as may be the case in the animals treated with L. H. and with A. P., eosinophilic granular cells would form but would not become vacuolated and would not produce androgen. Further, the absence of the first or of both factors may account for the finding that the ovaries of the animals treated with F. S. H. and P. U. contain cells which have neither vacuoles nor granules yet have an origin similar to that of the eosinophilic granular cells of the animals treated with P. M. S., L. H. and A. P. Whether the postulated factors are present in the material injected or whether they arise in the animal in response to the treatment is, of course, unknown. While the response under prolonged P. M. S. treatment supports the two-factor hypothesis, the possibility exists that since L. H., F. S. H., A. P. and P. U. cause the ovaries to become refractory, the ineffectiveness of these hormones in bringing about androgen production may be due to the onset of refractoriness before the necessary preliminary events are completed.

The luteal-like cells referred to in descriptions of ovaries transplanted to the ears of male mice (Hill, '37 a, b, and '41) are presumably identical with our eosinophilic interstitial cells, but since any epithelioid cells in the ovarian stroma are interstitial cells (Corner, '32), the more descriptive terms "vacuolated" and "unvacuolated" eosinophilic (interstitial) cells are used in the present paper in order to avoid a term which might connote a luteal function. The differences in histology between the ovaries which produced androgen and those which did not are of interest in view of Hill's ('41) report that he could find no consistent feature differentiating ovarian grafts which were active in the production of androgen. However, he did not describe vacuolated cells such as proved to be the distinguishing characteristic of androgen-producing ovaries in the present study. These cells, being simply a

further stage in development of normal interstitial cells, would undoubtedly be more conspicuous under the greater stimulation of P. M. S. treatment.

The fact that the interstitial cells in ovaries of mice of the A strain are derived from cells of the old corpora lutea which persist in situ or in dispersed nests of cells does not represent as great a divergence from the method of interstitial cell formation described for other forms as it might at first seem. In the cat and rabbit (Kingsbury, '39) the interstitial cells originate from the theca interna of atretic and old follicles, whereas in this strain of mice theca interna cells are carried into the new corpora lutea with the ingrowing blood channels and become indistinguishable from the granulosa cells. Probably both types of cells then participate in the formation of the nests of interstitial cells which develop eosinophilic granules, become vacuolated, and are associated with androgen production. The involvement of cells of the theca interna also corresponds to the observations that in rat ovaries growing in the ears of males (Deanesly, '38) and in guinea pig ovaries stimulated by hypophyseal extracts (Steinach and Kun, '31), androgen production is associated with thecal luteinization. Desclin's ('38) observation that masculinization of the rat did not occur when a pregnancy urine preparation was injected, despite the production of excessive luteal tissue, is identical with the results obtained on mice in the present study. The failure of excessive luteal tissue, as such, to produce androgen is in no way inconsistent with the finding that cells derived from corpora lutea are the probable source of this hormone. The luteal tissue is merely an early stage in the development of the cells apparently responsible for the production of the androgen. Also, it may be pointed out that if the rat ovary responds to pregnancy urine in the same way as does the ovary of the mouse, it is unlikely that masculinization would have occurred even with a more extended period of treatment.

While the identity of the androgen produced by the ovaries in this experiment is of course unknown, it is reasonably

certain that the androgenic action was not exerted by progesterone even though this substance has been reported to have androgenic properties (Greene, Burrill and Ivy, '39; Greene, Burrill and Thomson, '40; Lamar, '40). Such an action of progesterone has, on the other hand, been denied by Desclin ('39), and Hill ('37 a) did not observe stimulation of the seminal vesicles in castrated mice when 0.7 to 3.5 Allen-Corner rabbit units of progestin were administered either alone or in conjunction with estrogen. Similarly, in the present experiments no androgenic effects were observed in castrated male mice receiving progesterone in daily amounts up to 1.0 mg., which is equivalent to the maximum level the ovary can be made to produce by administration of P. M. S. (Pfeiffer and Hooker, '40). Further, the uterine histology and the survival after adrenalectomy (Pfeiffer and Hooker, '40) indicate that progesterone production reaches its peak while androgenic activity is still low, and subsides as the androgenic activity becomes more marked.

SUMMARY

1. Castrated male mice of the A strain (Strong, '36) bearing ovarian grafts in the ears and receiving 20 R. U. of P. M. S. daily from the time of grafting showed distinct stimulation of the seminal vesicles after 35 days of treatment. Smaller doses (10 R. U. or 5 R. U.) of P. M. S. caused the secretion of male hormone to a less degree and only after more prolonged treatment.

2. A pregnancy urine preparation (20 R. U. daily), whole anterior pituitary powder (sheep, 2 mg. daily), follicle stimulating hormone (2.5 R. U. daily), and luteinizing hormone (2 mg. daily) all failed to stimulate the production of male hormone by the ovary. However, the ovary usually became refractory in a short time.

3. Progesterone has been eliminated as the andromimetic substance produced by ovaries which stimulate the male accessory sex organs since injections of an amount of progesterone equivalent to that which the ovary is capable of

producing under P. M. S. treatment (1.0 mg. or less daily) did not maintain the accessories of castrated male mice of the A strain.

4. The interstitial cells in the ovaries of mice of the A strain are formed predominately from the breakdown of the corpora lutea.

5. The cells which secrete the androgen are thought to be vacuolated, epithelioid cells with eosinophilic granules which have the same origin as ordinary interstitial cells. That is, they are derived from the cells of the old corpora lutea and, as a result of ingrowth of the theca externa, become dispersed throughout the stroma in variously-sized cell masses.

6. The possibility is discussed that there are two factors involved in the development of the vacuolated, eosinophilic, granular cells; one controlling the growth and maturation of the cell (the formation of the granular cell) and the other governing the secretory phase (vacuolation).

LITERATURE CITED

- BRADBURY, J. T., AND F. GAENSBAUER 1939 Masculinization of female rats by gonadotropic extracts. *Proc. Soc. Exper. Biol. and Med.*, vol. 41, pp. 128-131.
- CORNER, G. W. 1932 Cytology of the ovum, ovary and fallopian tube. In *Cowdry's Special Cytology*, vol. 3, pp. 1567-1601.
- DEANESLY, R. 1938 Androgenic activity of ovarian grafts in castrated male rats. *Proc. Roy. Soc., London S. B.*, vol. 126, pp. 122-135.
- DESCLIN, L. 1938 A propos de l'activité androgénique de l'ovaire. *Compt. rend. Soc. de Biol.*, vol. 128, pp. 557-560.
- 1939 A propos de l'action androgénique de la progesterone. *Compt. rend. Soc. de Biol.*, vol. 132, pp. 43-45.
- EVANS, H. M., M. E. SIMPSON AND R. I. PENCHARZ 1937 An anterior pituitary gonadotropic fraction (ICSH) specifically stimulating the interstitial tissue of testis and ovary. *Symposia on Quantitative Biology*, vol. 5, pp. 229-240.
- GARDNER, W. U. 1935 The effect of ovarian hormones and ovarian grafts upon the mammary glands of male mice. *Endocrinology*, vol. 19, pp. 656-667.
- GOODMAN, L. 1934 Observations on transplanted immature ovaries in the eyes of adult male and female rats. *Anat. Rec.*, vol. 59, pp. 223-252.
- GREENE, R. R., AND M. W. BURRILL 1939 a Experimental intersexuality; masculinization of female rats by post partum treatment with anterior pituitary-like hormones. *Proc. Soc. Exper. Biol. and Med.*, vol. 40, pp. 514-516.

- GREENE, R. R., AND M. W. BURRILL 1939 b Androgenic function of APL stimulated ovaries in immature rats. *Proc. Soc. Exper. Biol. and Med.*, vol. 42, pp. 761-764.
- GREENE, R. R., M. W. BURRILL AND A. C. IVY 1939 Progesterone is androgenic. *Endocrinology*, vol. 24, pp. 351-357.
- GREENE, R. R., M. W. BURRILL AND D. M. THOMSON 1940 Further studies on the androgenicity of progesterone. *Endocrinology*, vol. 27, pp. 469-472.
- GUYÉNOT, E., AND MME. DUSZYNSKA-WIETRZYKOWSKA 1935 Stérilité et virilisme chez des femelles de cobayes issues d'un croisement interspécifique. *Rev. Suisse de Zool.*, T. 42, pp. 341-388.
- GUYÉNOT, E., AND I. NAVILLE-TROLLET 1936 Masculinisation provoquée de femelles de cobayes (Extraits hypophysaires et urine de femmes enceintes). *Rev. Suisse de Zool.*, T. 43, pp. 415-454.
- GUYÉNOT, E., K. PONSE, E. DOTTERNS, M. VALLETTE AND J. TROLLET 1933 Action des extraits alcalins d'hypophyses (lobes antérieurs) sur le Cobaye. *Rev. Suisse de Zool.*, T. 40, pp. 217-222.
- GUYÉNOT, E., K. PONSE AND I. TROLLET 1934 Action masculinisante de l'urine de femme enceinte. *Compt. rend. de l'Acad. Sci.*, T. 198, pp. 1830-1832.
- GUYÉNOT, E., K. PONSE AND J. WIERZYKOWSKA 1932 Lutéinisation de l'ovaire et masculinisation chez le Cobaye. *Compt. rend. de l'Acad. Sci.*, T. 194, pp. 1051-1053.
- HILL, R. T. 1937 a Ovaries secrete male hormone, restoration of castrate type of seminal vesicles and prostate glands to normal by grafts of ovaries in mice. *Endocrinology*, vol. 21, pp. 495-502.
- 1937 b Ovaries secrete male hormone; temperature control of male hormone output by grafted ovaries. *Endocrinology*, vol. 21, pp. 633-636.
- 1941 Fate of ovaries which have been grafted in the ear for long periods of time. *Endocrinology*, vol. 28, pp. 426-430.
- HILL, R. T., AND W. U. GARDNER 1936 Maintenance of accessory organs by ovarian grafts into castrate male mice. *Anat. Rec.*, vol. 64, (suppl. 3), p. 21.
- HILL, R. T., AND M. T. STRONG 1938 Ovaries secrete male hormone. IV. Effects of ovarian androgens on accessory size in the mouse. *Endocrinology*, vol. 22, pp. 663-666.
- HOOKE, C. W. 1940 Effects of progesterone upon the uterus of the mouse. *Proc. Soc. Exper. Biol. and Med.*, vol. 45, pp. 270-272.
- DE JONGH, S. E., AND R. KORTEWEG 1935 Der Einfluss von Ovar-implantationen auf die Genitalien der kastrierten männlichen Maus. *Acta Brevia Neerl.*, vol. 5, pp. 126-127.
- KINGSBURY, B. F. 1939 Atresia and the interstitial cells of the ovary. *Am. J. Anat.*, vol. 65, pp. 309-331.
- LAMAR, J. K. 1940 Some effects of corpus luteum extracts and of synthetic progesterone on young male albino rats. *Physiol. Zool.*, vol. 13, pp. 251-267.

- LIPSCHÜTZ, A. 1932 Weidervermännlichung eines kastrierten männlichen Meer-schweinchens nach Eierstocksverpflanzung. *Virchows Arch. f. path. Anat.*, Bd. 285, S. 35-45.
- 1933 Masculinisation par résection partielle de l'ovaire chez le cobaye. *Compt. rend. Soc. de Biol.*, T. 112, pp. 1272-1274.
- 1937 Androgenic endocrine activity in female mammals. *Nature*, London, vol. 140, p. 892.
- PAPANICOLAOU, G. N., AND E. A. FALK 1934 Action of pregnancy urine extract (follutein) on external genitalia of female guinea pigs. *Proc. Soc. Exper. Biol. and Med.*, vol. 31, pp. 750-751.
- PFEIFFER, C. A. 1936 Sexual differences of the hypophyses and their determination by the gonads. *Am. J. Anat.*, vol. 58, pp. 195-225.
- 1939 The effects of an experimentally induced endocrine imbalance in female mice. *Anat. Rec.*, vol. 75, pp. 465-491.
- PFEIFFER, C. A., AND C. W. HOOKER 1940 Hormonal factors affecting the survival of adrenalectomized mice. *Am. J. Physiol.*, vol. 131, pp. 441-448.
- 1942 Effects of chronic pregnant mare serum treatment in female mice of the A strain. (In press.)
- STEINACH, E., AND H. KUN 1931 Luteingewebe und männliche Geschlecht-scharaktere. *Arch. f. d. ges. Physiol.*, vol. 227, pp. 266-278.
- STRONG, L. C. 1936 Establishment of "A" strain of inbred mice. *J. Hered.*, vol. 27, pp. 21-24.

PLATE 1

EXPLANATION OF FIGURES

All figures are photomicrographs of tissues from mice of the A strain (Strong, '36) prepared by the paraffin method, sectioned at 8μ and stained with Ehrlich's hematoxylin and triosin.

1 Ovarian graft after 35 days residence in the ear of a castrated male which had been receiving 20 R. U. of P. M. S. daily. Note the organized corpus luteum (lower center) and the older corpus luteum which is being broken up by ingrowths of the theca externa (upper left) to form the smaller masses of altered cells (eosinophilic granular cells) which may be seen scattered throughout the stroma. $\times 100$.

2 Ovarian graft after 60 days residence in the ear of a castrated male which had been receiving 20 R. U. of P. M. S. daily. Almost the entire graft now consists of eosinophilic granular cells. $\times 100$.

3 Seminal vesicle of the castrated male which carried the graft shown in figure 1. Note the tall columnar epithelium and the numerous secretory granules. $\times 175$.

4 Seminal vesicle of the castrated male which carried the ovarian graft shown in figure 2. Note the columnar epithelium, secretory granules and accumulation of normal secretion in the lumen. $\times 200$.

5 Ovary after 60 daily injections of 20 R. U. of P. U. The organization of the cells is somewhat similar to that found after treatment with P. M. S., but the cells differ from those in ovaries of P. M. S. treated animals in that they have pale non-granular cytoplasm and their nuclei are typically less vesicular. (Compare with figs. 1 and 2). $\times 200$.

6 Ovary after 100 days treatment with 20 R. U. of P. U. daily. The cell masses are more conspicuously delineated by ingrowths of connective tissue from the theca externa than at 60 days, and the nuclei of the cells are often pycnotic. In other respects the histology and cytology of the ovary is similar to that shown in figure 5. $\times 300$.

7 Seminal vesicle of a castrated male which had been carrying an ovarian graft in the ear and had been receiving 20 R. U. of P. U. daily for 100 days. Note that while the epithelium is of practically the castrate type, there is some secretion and distention of the lumen with a clear fluid unlike the normal secretion of the seminal vesicle. $\times 200$.

8 Seminal vesicle of a castrated male which had been carrying an ovarian graft in the ear and had been receiving daily injections of 2 mg. of unfractionated, acetone-dried, whole sheep pituitary powder for 90 days. Note the completely castrate condition of the epithelium. $\times 300$.

9 Ovary after 90 days of treatment with 2.5 R. U. of F. S. H. daily. The corpus luteum is composed of typical luteal cells, but the cells in the masses derived from older corpora lutea are relatively agranular and have pycnotic nuclei. $\times 100$.

10 Ovary after 90 days treatment with 2 mg. of L. H. daily. The small masses of altered cells are not numerous, but the cells contain eosinophilic granules and have vesicular nuclei. They never become vacuolated as under P. M. S. treatment. $\times 100$.

11 Seminal vesicle of a castrated male which had carried an ovarian ear graft and had received 2.5 R. U. of F. S. H. daily for 90 days. Note the castrate condition of the epithelium. $\times 300$.

12 Seminal vesicle of a castrated male which had carried an ovarian ear graft and had received 2 mg. of L. H. daily for 90 days. Note the castrate condition of the epithelium. $\times 300$.

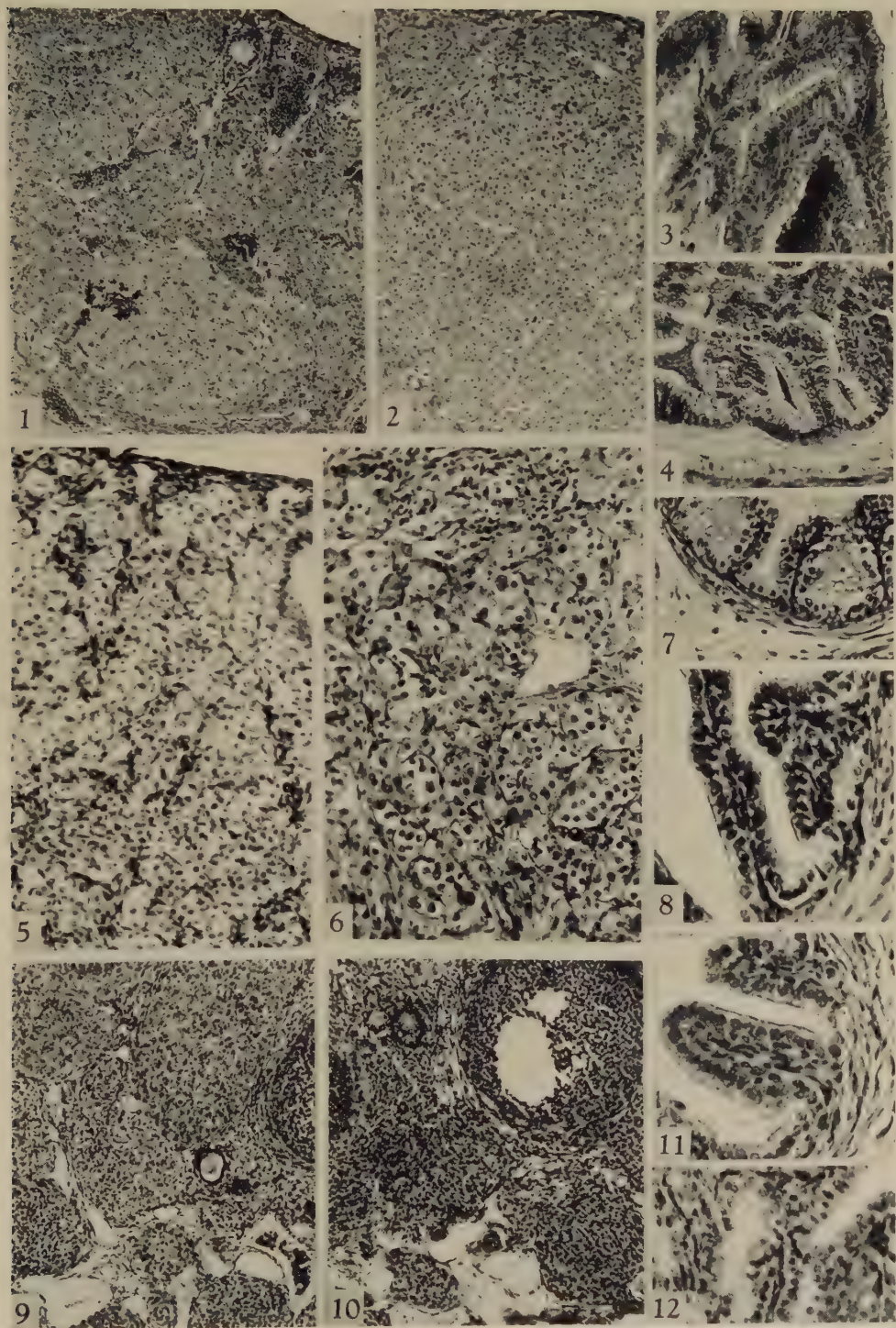


PLATE 2

EXPLANATION OF FIGURES

All figures are photomicrographs of tissues from mice of the A strain (Strong, '36) prepared by the paraffin method, sectioned at 8μ and stained with Ehrlich's hematoxylin and triosin.

13 Ovarian follicle from a normal mouse of the A strain about 12 hours after ovulation. This is an entirely normal picture of early corpus luteum formation. $\times 150$.

14 Ovary of a normal mouse of the A strain about 24 hours after ovulation. This is about the maximum degree of development attained by the corpus luteum in the non-pregnant female. The interstitial cells are well developed. $\times 150$.

15 Portion of a young follicle from the ovary of a mouse which had received 20 R. U. of P. M. S. daily for 4 days. Note the beginning thecal luteinization. $\times 300$.

16 Portion of a well organized older corpus luteum from a mouse which had received 20 R. U. of P. M. S. daily for 25 days. Arrows designate the points at which the theca externa is beginning to grow in to break up the corpus luteum into smaller masses of cells. $\times 300$.

17 Ovary after 35 days treatment with 20 R. U. of P. M. S. daily. Note the accumulation of eosinophilic granular cells. These cells somewhat resemble the luteal cells, but they become vacuolated and secrete male hormone. $\times 150$.

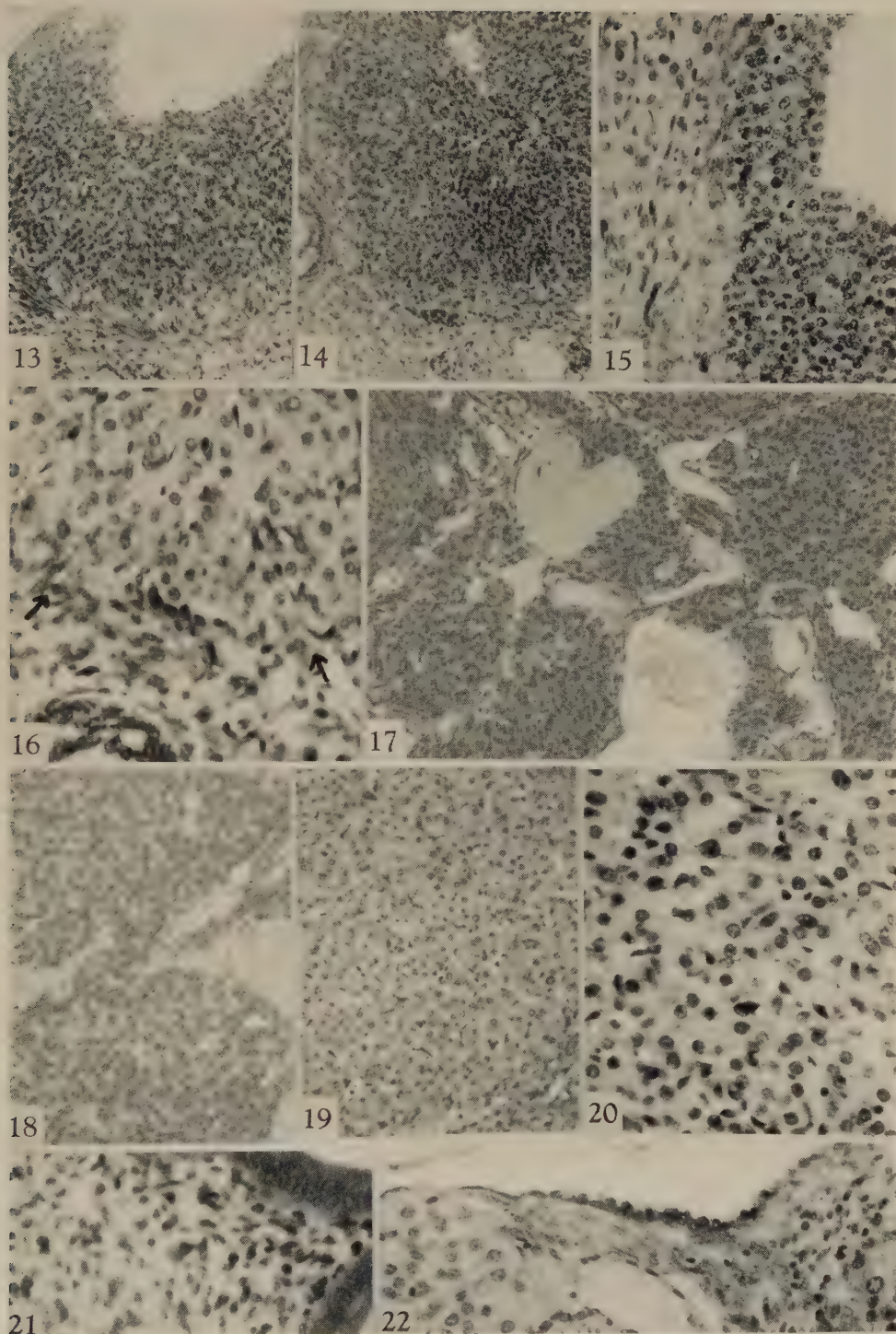
18 Ovary after 53 days treatment with 20 R. U. of P. M. S. daily. The eosinophilic granular cells are becoming less granular and more vacuolated. The vascularity of the ovary is greatly increased. $\times 150$.

19 A portion of an old corpus luteum from a mouse which had received 20 R. U. of P. M. S. daily for 60 days, showing the break-up of the organized corpus luteum into cell masses. $\times 150$.

20 High power view of the eosinophilic granular cells in the ovary of a mouse which had received 20 R. U. of P. M. S. daily for 65 days. Almost all of these cells are now appreciably vacuolated, and the cytoplasm is only slightly granular. However, there are a few cells present, characterized by enlarged ellipsoid to round nuclei, which contain eosinophilic granules but are not vacuolated. $\times 300$.

21 Endometrium of the uterus after 35 days treatment with 20 R. U. of P. M. S. daily. Note the fibrous nature of the tunica propria which indicates the action of male hormone. $\times 300$.

22 Small portion of an ovary after 60 days treatment with 20 R. U. of P. M. S. daily, demonstrating that small patches of germinal epithelium can still be found at the periphery of the ovary between the masses of eosinophilic granular cells. Note the marked vacuolation of many of the eosinophilic granular cells.



AN ANALYSIS IN HUMAN SEMEN OF A STAINING METHOD FOR DIFFERENTIATING LIVE AND DEAD SPERMATOOZOA

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In a recent publication Lasley, et al. ('42) described a new staining technique for determining the number of live and dead spermatozoa in ram semen. The technique is based on their observation that "various stains would enter certain spermatozoa but not others" and that "in every case it was observed that the sperm in which the stain entered were non-motile before and after staining."² The photomicrographs made by Lasley, et al. of ram spermatozoa stained under these conditions show a sharp differentiation in staining properties, the "dead" cells staining darkly while the "live" cells show no evidence of dye penetration. In view of the fact that determination of the number of viable cells in human semen is subject to considerable error, it is essential that any suggestion of a new technique for estimating the ratio of live to dead cells in the human ejaculate be given the most rigorous examination. It is generally assumed that in the absence of morphological defects motility is the best criterion of the potential fertility of the spermatozoon but it does not follow that a cell lacking motility in the semen specimen is not capable of motility in another environment. In other words, motility is not necessarily the only criterion of viability as has been shown in studies on the revival of motility in spermatozoa rendered inert by various methods (Shettles,

¹ Aided by a grant from the National Committee on Maternal Health.

² The stain is a mixture of water soluble eosin and opal blue. Doctor Lasley was kind enough to send me a sample of the stain for trial on human material.

'40; MacLeod, '41). Therefore, any technique designed to differentiate between non-viable and viable spermatozoa must include enumeration of the potentially motile as well as the motile cells. Lasley and his co-workers believe that their stain satisfies these requirements for ram spermatozoa.

The following report is based on a study of normal human spermatozoa under a variety of experimental conditions and using the stain and technique advocated by Lasley and his co-workers. It will be shown that, in human spermatozoa, a different interpretation can be placed on the staining differentiation seen in these cells. As in Lasley's experiments, emphasis is placed on the relation between the number of motile cells seen in any given specimen and the number of cells which remain unstained, with the reservation that lack of motility does not necessarily imply non-viability.

METHODS

The semen was delivered into glass receptacles and received in the laboratory within an hour after ejaculation. The specimens were examined immediately for the total number of spermatozoa and for motility, using methods described in detail elsewhere (MacLeod, '41). The method of estimating the number of motile cells is admittedly crude and probably errs on the low side but, after examining several thousand normal semen specimens, we believe the error to be no greater than 10%.

The staining properties of the motile cells were then observed in the living preparation by adding a small amount of stain to a drop of semen, mounting with cover-glass, and examining the active and inert cells under high dry power. Stained dry smears were then made and 200 cells examined on each smear. The study was extended by changing the pH of the semen in such a way that the motility of the cells was completely depressed by acidification and restored again by neutralization of the medium. The pH measurements were made on the Beckman glass electrode, 1% lactic acid being used for acidification and sodium bicarbonate for neutraliza-

tion. Stained smears of the spermatozoa were made when motility was completely depressed and again when motility was restored.

Lastly, the staining properties of the spermatozoa were studied in Ringer's solution under various experimental conditions given in detail below.

OBSERVATIONS

Table 1 gives the data derived from twenty-six experiments in which the number of motile sperm in fresh human semen is compared with the number of unstained cells in stained preparations prepared immediately after the motility determinations were made. The figures show that while only 54% of

TABLE 1
Summary of the motility and staining properties of twenty-six specimens of normal human spermatozoa.

NUMBER OF EXPERIMENTS	MEAN AGE OF SPECIMEN (HOURS)	MEAN % OF MOTILE CELLS	MEAN % UNSTAINED SPERMATOZOA ("LIVE")	MEAN % STAINED SPERMATOZOA ("DEAD")
26	1.5	54	83	17

the cells are motile just prior to staining, 83% are "alive" according to the staining criterion. As was mentioned above, the method of estimating motility probably errs, at the most, about 10% on the low side in which event the staining technique indicates a considerably higher percentage of viable cells. When the staining properties of the motile cells in the living preparation are examined, it is seen that whereas a few of the inert cells take the stain, a much higher number remain unstained. It is much more difficult to determine if the fast-moving active cells are stained since, in the wet preparation, the greatest degree of staining is only a faint pink and this can be seen clearly only when the cells remain stationary in the field. However, if one assumes that the motile cells are not stained, there still remains the question of differentiating between the stained and unstained inert cells in the wet

preparation. According to Lasley, et al. the unstained inert cells are alive and potentially motile whereas the stained inert cells are dead. It will be shown below that this definition does not hold in all cases.

Effect of varying pH of semen

Table 2 demonstrates the variations in the staining reaction of the spermatozoa which can be induced by varying the pH and alternately depressing and restoring motility. These results can be summarized briefly. When the pH of the semen is lowered to 5.9 by adding lactic acid, motility ceases. When

TABLE 2
The effect of semen pH on the motility and staining properties of human spermatozoa.

pH	% MOTILE CELLS	% UNSTAINED CELLS ("LIVE")	% STAINED CELLS ("DEAD")
7.5	53	92	8
5.9	no motility	49	51
7.9	47	86	14
5.9	no motility	31	69
7.8	45	65	35

All data obtained from same specimen of semen.

the pH is restored to its original level by adding bicarbonate, maximal motility is also restored in the same number of cells originally active in the untreated semen. However, when stained smears are made at each stage of the acidification and neutralization, it is obvious from the figures in the "stained cells" column (table 2) that most of the spermatozoa which stained "dead" at pH 5.9 when all motility was depressed, recovered their motility and their ability to prevent entrance of the stain at pH 7.9. Under these experimental conditions, therefore, the ability of the stain to enter human spermatozoa is reversible and the presence of the stain within the cells cannot be interpreted as meaning that such cells are "dead."

*The effect of Ringer's solution on the staining properties
of human spermatozoa*

When human spermatozoa are transferred to Ringer's solution motility is not unduly affected and is maintained maximally for many hours at 38°C. (MacLeod, '41). When these cells in this medium are stained according to the above technique, the stain penetrates every cell almost without exception, in spite of the fact that at least 50% of the spermatozoa show maximal motility just prior to staining. If to a drop of the spermatozoa suspension in Ringer's solution is added an equal amount of cell-free seminal fluid, approximately 65% of the spermatozoa remain unstained, another example of the reversible nature of the staining phenomenon. It is not the purpose of this paper to analyze the mechanism of the staining reactions involved. Rather is it merely to point out that by varying the experimental conditions almost any result can be obtained, none of which can properly be interpreted as differentiating between "live" and "dead" spermatozoa.

DISCUSSION

In view of the above results, the staining technique advocated by Lasley, et al. for ram semen cannot be applied to human spermatozoa. The staining reactions of the spermatozoa are influenced by factors other than the ability of the cells to perform functions usually associated with a live, functioning organism. The results obtained from the spermatozoa in Ringer's solution in which the spermatozoa all stain "dead" in spite of their maximal and sustained motility may be explained on the basis of a change in permeability of the cells due to transference from one medium to another. This explanation is reasonable but whatever properties of the spermatozoa are changed in Ringer's solution, the fact remains that their metabolic activity remains high in this medium (MacLeod, '41; Ross, et al. '41), and their motility relatively unimpaired. It is not possible at present to say that human spermatozoa retain their fertilizing power in a

balanced salt medium but it should be pointed out that artificial insemination in farm live-stock and in other animals is performed successfully using semen diluted with various modifications of a balanced salt solution (Berliner, et al. '38; Lambert and McKenzie, '40). It is possible, however, that the spermatozoa of other mammals, e.g., the ram, may not change their staining properties when subjected to certain experimental procedures. Comparative studies of this nature will be of great value in furthering our knowledge of the biology of mammalian spermatozoa.

SUMMARY

1. A new staining technique for differentiating between live and dead ram spermatozoa has been analyzed in relation to human spermatozoa.

2. The results show that the differentiation in staining properties seen in human spermatozoa using this technique cannot be interpreted as distinguishing between live and dead cells.

LITERATURE CITED

- LASLEY, J. F., G. T. EASLEY AND F. F. MCKENZIE 1942 A staining method for the differentiation of live and dead spermatozoa. *Anat. Rec.*, vol. 82, pp. 167-173.
- MACLEOD, J. 1941 Effect of glycolysis inhibitors and of certain substrates on the metabolism and motility of human spermatozoa. *Endocrinol.*, vol. 29, pp. 583-591.
- SHETTLES, L. B. 1940 The respiration of human spermatozoa and their responses to various gases and low temperatures. *Amer. J. Physiol.*, vol. 128, pp. 408-415.
- ROSS, V., E. G. MILLER, JR. AND R. KURZROK 1941 Metabolism of human sperm. *Endocrinol.*, vol. 28, pp. 885-893.
- BERLINER, V. R., F. E. COWART, R. H. MEANS AND J. B. WRIGHT 1938 Artificial insemination of horses and jennets for horse, mule, and jack-stock production. *Proc. Am. Soc. Animal Reprod.*, 1938.
- LAMBERT, W. V., AND F. F. MCKENZIE 1940 Circular no. 567. U. S. Dept. of Agric.

EFFECT OF TESTOSTERONE PROPIONATE ON SPERMATOGENESIS IN HYPOPHYSECTOMIZED RATS FOLLOWING THE INJECTION OF GONADOTROPINS ¹

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ONE PLATE (SIX FIGURES)

It has been shown that spermatogenesis can be maintained in hypophysectomized rats with androgenic substances (Walsh, Cuyler and McCullagh, '34; Nelson and Gallagher, '36; Cutuly, McCullagh and Cutuly, '37), sperm being present in the testes after 178 days of treatment (Nelson, '40). Recently, Nelson ('41) has reported that spermatogenesis can be restored in rats following a postoperative interval of 22 to 28 days provided 2 to 3 mg. daily doses of testosterone propionate are used. Similarly, spermatogenesis can be maintained or restored after hypophysectomy with extracts of the urine of men (Wells and Overholser, '38; Leatham, '40; Leatham and Mills, '40) or with pregnant mare serum (Evans et al., '33; Smith, '35; Greep, '37; Liu and Noble, '39). It appeared of interest to investigate the effect of an androgen on the testes of hypophysectomized rats after spermatogenesis had been restored with gonadotropic materials.

For these experiments, adult male rats, 97 to 158 days old were hypophysectomized. All pituitary capsules were serially

¹ Aided by a grant from the Rockefeller Foundation, New York, administered by Dr. P. E. Smith.

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sectioned, stained and examined microscopically. Data from completely hypophysectomized rats only are presented.

Spermatogenesis was maintained or restored by the daily subcutaneous injection of either 2 rat units (Cartland-Nelson) of pregnant mare serum, Gonadogen, or 1-2 mg. of male urine extract Prospermin.³ To verify the activity of each of these extracts on spermatogenesis groups of five hypophysectomized rats were used. Following a postoperative interval of 30 to 32 days, the animals were then injected for 25-33 days, after which time spermatozoa were present in the testes in all cases. Testis weight averaged 1339 mg. following treatment with PMS and 1549 mg. following treatment with male urine extract.

The experiments involving the use of testosterone propionate were divided into two groups. In the first group injections of the gonadotropic extract was started the day following hypophysectomy. Thirty days later one testis was removed from each of the seven animals. Histological examination of the testes revealed the presence of spermatozoa in all cases as well as a moderate increase in interstitial tissue (fig. 5). Spermatozoa were present in the epididymides. The administration of testosterone propionate was then started in four rats and continued for 35 days; no treatment was given to three other animals during this period. All the rats were sacrificed the day following the cessation of treatment. A slight regression in testis weight occurred in the androgen treated rats but spermatogenesis was well maintained in all cases and sperm were present in the epididymides. The interstitial tissue had regressed markedly (fig. 6). On the other hand, in the animals not treated with androgen, no spermatozoa were present in either the testes or the epididymides and the anticipated regression in testis weight occurred (table 1).

³ The writer wishes to express his appreciation for the extracts used in this investigation to the following: Dr. G. F. Cartland of the Upjohn Company for the pregnant mare serum, Gonadogen, Dr. J. A. Morrell of E. R. Squibb & Sons for the male urine extract, Prospermin and to Drs. E. Oppenheimer and R. Mautner of Ciba Pharmaceutical Products for the testosterone propionate.

To test the fertility of the androgen treated animals, two males, having been injected for 30 days, were caged with females in estrus. In each case copulation took place and normal litters were sired.

In the second group (b, table 1), a postoperative interval of 20 days was allowed to elapse after hypophysectomy before beginning treatment with gonadotropic hormone, since it is known that regression of the testes occurs within 20 days after hypophysectomy. In this group of animals the testes of the seventeen untreated rats were devoid of spermatozoa.

TABLE 1

Effect of testosterone propionate on spermatogenesis in hypophysectomized rats following injection of gonadotropins.

NO. OF RATS	AVE. WT. TESTIS A ¹	TREATMENT	AVE. WT. TESTIS B	AVE. WT. SEM. VES.
(a) Immediate treatment with gonadotropin for 30 days				
	mg.		mg.	mg.
4	1332	1 mg. T.P. $\times$ 35	1050	2622
3	1335	None	348	171
(b) Treatment delayed 20 days followed by gonadotropin for 30 days				
6	784	1 mg. T.P. $\times$ 20	832	2148
4	731	None	296	160
17	Untreated rats 20 days after hypophysectomy		640 ²	117
20	Normal controls		2473 ²	910

¹ Testis A removed day following last injection of gonadotropin.

² Weight of two testes.

Restoration of spermatogenesis was attempted in ten rats by the injection of gonadotropic extracts for 30 days, one testis from each rat then being removed. Spermatozoa were present in all cases but the size of the seminiferous tubules and the apparent number of spermatozoa present was reduced in comparison with unoperated control animals (figs. 1 and 3). There was a large number of spermatozoa in the epididymides of one animal whereas in three rats there was none. Daily

subcutaneous administration of 1 mg. of testosterone propionate was then started in six rats and continued for 20 days. Four rats were allowed to go untreated for the 20-day period. All rats were sacrificed the day following the last injection. Testis weight was maintained in the androgen treated rats and the seminiferous tubules contained spermatozoa in all cases, tubular size and the apparent number of spermatozoa being well maintained (fig. 2). Many spermatozoa were present in the epididymis of the one animal in which spermatozoa had previously been seen contralaterally, and two other rats had a few spermatozoa in the epididymis.

The testes and the epididymides of the rats not receiving androgen were devoid of spermatozoa (fig. 4).

The seminal vesicles were abnormally large in the androgen treated animals.

SUMMARY

Spermatogenesis which has been maintained or restored with gonadotropic hormones following hypophysectomy is maintained with testosterone propionate. Fertile matings are obtained under the experimental conditions cited.

LITERATURE CITED

- CUTULY, E., D. R. McCULLAGH AND E. C. CUTULY 1937 Effects of androgenic substances in hypophysectomized rats. *Am. J. Physiol.*, vol. 119, pp. 121-126.
- EVANS, H. M., K. MEYER AND M. E. SIMPSON 1933 The growth and gonad-stimulating hormones of the anterior hypophysis. *Mem. Univ. Calif.*, vol. 11.
- GREEP, R. O. 1937 Pituitary regulation of the male gonad. *Cold Spring Harbor Symposium*, vol. 5, pp. 136-144.
- LEATHEM, J. H. 1940 Effect of "Prospermin" on immature and mature hypophysectomized and normal male rats. *Proc. Soc. Exp. Biol. and Med.*, vol. 45, pp. 497-499.
- LEATHEM, J. H., AND E. J. MILLS, JR. 1940 Influence of normal male urine gonadotropin on spermatogenesis in hypophysectomized immature rats. *Anat. Rec. (suppl.)*, vol. 78, p. 108.
- LIU, S. H., AND R. L. NOBLE 1939 The effects of extracts of pregnant mare serum and human pregnancy urine on the reproductive system of hypophysectomized male rats. *J. of Endocrinology*, vol. 1, pp. 7-14.

- NELSON, W. O., AND T. F. GALLAGHER 1936 Some effects of androgenic substances in the rat. *Science*, vol. 84, pp. 230-232.
- NELSON, W. O. 1940 Maintenance of spermatogenesis in hypophysectomized rats. *Am. J. Physiol.*, vol. 129, p. P430.
- 1941 Renewal of sperm formation in hypophysectomized rats. *Anat. Rec. (suppl.)*, vol. 79, p. 48.
- SMITH, P. E. 1935 The hypophyseal gonadotropic hormones. *J. Am. Med. Assoc.*, vol. 104, pp. 553-556.
- WALSH, E. L., W. K. CUYLER AND D. R. McCULLAGH 1934 The physiologic maintenance of the male sex glands. *Am. J. Physiol.*, vol. 107, pp. 508-512.
- WELLS, L. J., AND M. D. OVERHOLSER 1938 Sperm formation and growth of accessory reproductive organs in hypophysectomized ground squirrels in response to substances from blood and urine. *Anat. Rec.*, vol. 72, pp. 231-248.

PLATE 1

EXPLANATION OF FIGURES

1 Seminiferous tubules of an adult rat (GH22844) which had been hypophysectomized for 50 days and treated with 2 units of PMS daily during the last 30 days. Spermatozoa present. $\times 120$.

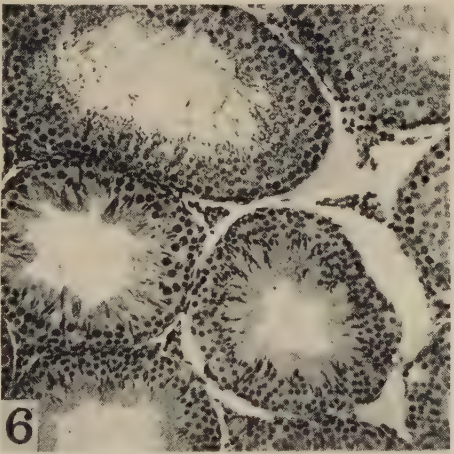
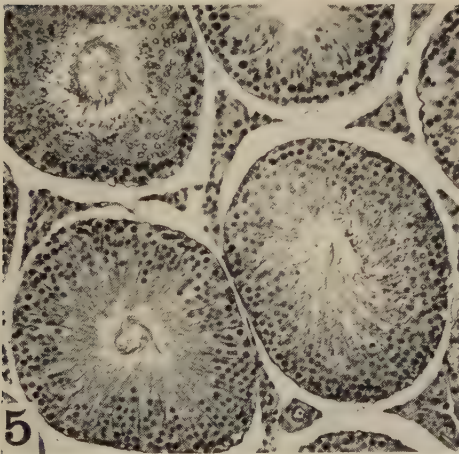
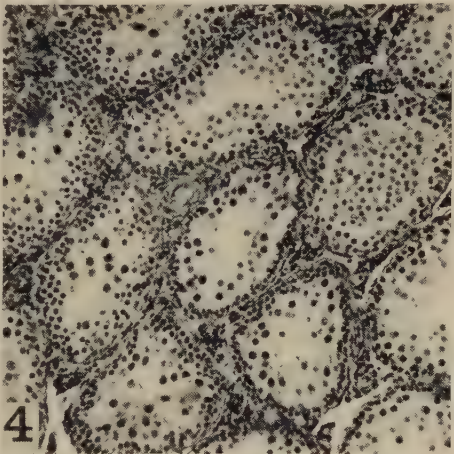
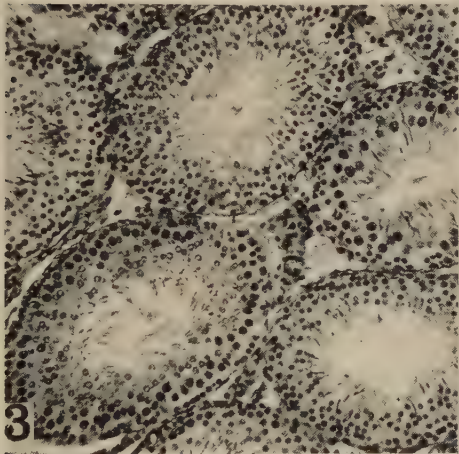
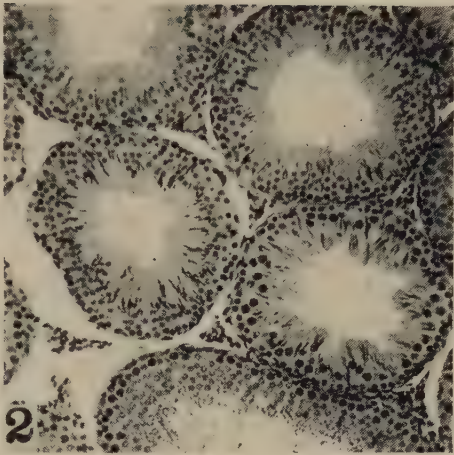
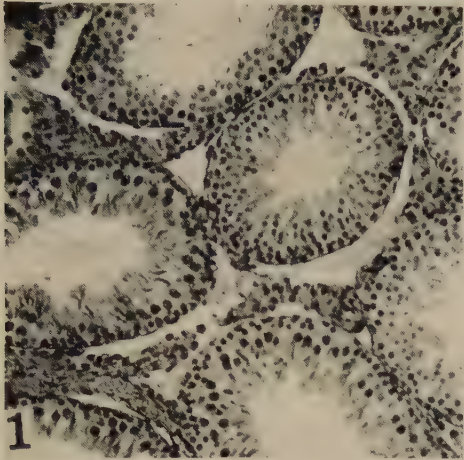
2 Seminiferous tubules of rat GH22844 in which the restored spermatogenesis was maintained for an additional 20 days with 1 mg. of testosterone propionate daily. $\times 120$.

3 Seminiferous tubules of an adult rat (G22910) which had been hypophysectomized for 50 days and treated with 1 mg. of male urine extract daily during the last 30 days. Spermatozoa present. $\times 120$.

4 Seminiferous tubules of rat 22910 in which treatment was discontinued for 20 days after the restoration of spermatogenesis. No spermatids or spermatozoa. $\times 120$.

5 Seminiferous tubules of an adult rat (G22844). Spermatogenesis was maintained for 30 days with 1 mg. of male urine extract daily. $\times 120$.

6 Seminiferous tubules of rat 22844. Following the maintenance of spermatogenesis with Prospermin, maintenance of spermatogenic function was continued for 35 days with 1 mg. of testosterone propionate daily. $\times 120$.



A STATISTICAL STUDY OF THE NUMBER OF WHITE BLOOD CELLS IN THE BLOOD OF THE RABBIT ^{1, 2}

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Many studies have been made to determine the numbers of white blood cells in the rabbit. Frequently in these the number of both animals and counts were small. Furthermore, all of the data were not considered statistically. Some investigators have determined the number of white blood cells in the rabbit under specific conditions by statistical methods. Pearce and Casey ('30) made such a study on 1110 counts on 174 normal male rabbits. Their experiment extended over a period of 1 year. The counts were made at approximately the same hour of the day and not more often than twice in 1 week. To establish the mean for rabbits it seems advisable to include the results of this experiment with those of others in which the cells from female rabbits are also counted. The counts should be made at more frequent intervals than only twice a week since the cells of rabbits are counted more often than this in most experimental procedures.

We are reporting in this paper the results of 580 counts made upon 165 normal rabbits. These data are analyzed by standard statistical methods to determine, first the normal white blood cell count for the rabbit and second, the fluctuation in the count during the day. We are also including in this study a statistical analysis of a large number of counts recorded in the literature by other investigators.

¹ Aided by grants from the John and Mary R. Markle Foundation and the University of Tennessee.

² I am greatly indebted to Dr. F. L. Roberts, Professor of Preventive Medicine, University of Tennessee for his assistance in the statistical problems concerned in this study.

METHODS AND MATERIALS

The rabbits were adults of both sexes, weighing from 1–3 K. They were mixed breeds and raised by several different animal breeders. The animals were brought into the laboratory at intervals varying from immediately to several days before the white cells were counted for the first time. They were kept in separate cages and fed cabbage, oats and given water ad lib. This experiment extended over a period of 1 year. The animals were given satisfactory care and their quarters were steam heated during the winter months.

Blood for the counts was obtained by puncturing the veins in the lateral margins of the ears with a knife. No irritants were applied to the skin of the ear of the rabbits used in this study. Xylol was carefully applied, however, to the ear of a second group of rabbits to determine whether or not it effected the number of circulating white blood cells. The blood used in these counts was diluted with a 1.0% solution of acetic acid. A standard hemacytometer and pipettes were used. The pipettes were shaken by hand for approximately 3 minutes and the cells were counted immediately thereafter. All counts were made by one of us (A. H.).

The blood for these counts was taken from the ears at varying times during the day. This time is given in the different experiments. The number of counts made upon each rabbit and also the number of rabbits used in each series is given in the different experiments.

EXPERIMENTAL

A total of 580 white blood cell counts were made upon 165 rabbits. There were 29 rabbits upon which one count was made, 17 rabbits upon which three counts were made, 104 rabbits upon which four counts were made, 12 rabbits had five counts each and 3 rabbits had eight counts each.

The distribution of these counts is not Gaussian, as is shown by the fact that Beta_1 is equal to 1.38 and Beta_2 is equal to 4.25. Since the distribution is not Gaussian or

"normal" very little meaning is attached to the standard deviation of this distribution. Accordingly the deciles were calculated and this calculation showed that 90% of the counts were above 6530 and 90% of the counts were below 16,148. Eighty per cent of the counts were between 6,530 and 16,148 and more than one-half of the counts (60%) were between 7,320 and 13,600.

White blood cell counts were made at specific hours during the day to determine if there was any variation in the counts in the forenoon and afternoon. The counts as shown in table 1 were made at 9 A.M. and 1 P.M. on sixty-two rabbits. Counts were also made at 3 P.M. on fifty-four of these sixty-two rabbits. The coefficients of correlation between the counts and the time of day were .5082 for the 9 A.M. and 1 P.M. counts and .3611 for the 9 A.M. and the 3 P.M. counts.

TABLE 1

Differences in leucocyte counts on rabbits at various hours of the same day.

BETWEEN	NO. OF RABBITS	NO. OF COUNTS	DIFF. IN MEANS	STANDARD ERROR OF DIFFERENCE	DIFF.
					S.E. diff.
9 A.M. and 1 P.M.	62	62	1933	531	3.64
9 A.M. and 3 P.M.	54	54	1702	532	3.08

It was necessary to calculate these coefficients in order to get the standard error of the difference in means of the forenoon and the afternoon counts. There was a statistically significant increase in the mean count at 1 P.M. and 3 P.M. over the mean count at 9 A.M.

The following data are given also to show the variation in the cell count during the forenoon and the afternoon. Counts were made upon thirty rabbits at 9:00 A.M.; 11:00 A.M.; 1:00 P.M. and 3:00 P.M. as shown in table 2. From these data it is evident that there is an increase in the afternoon count over that of the forenoon; however, in this sample it is not possible to demonstrate that the difference is statistically significant. In a second group of twenty rabbits the leucocyte counts were

made at 9:00 A.M., 1:00 P.M. and 3:00 P.M. with no count at 11:00 A.M. These rabbits show a statistically significant increase of the mean count at 1:00 P.M. over the mean count at 9:00 A.M.; however, there is no significant difference between the counts made at 9:00 A.M. and 3:00 P.M. These observations would indicate that any inflammation that may result from the pricking of the ear apparently has no effect upon the number of circulating leucocytes since the rabbits shown in table 2 have an intervening count at 11:00 A.M. and there is no demonstrable significant increase, whereas in the second group of twenty rabbits with no intervening count at 11:00 A.M. there is a significant increase.

TABLE 2
Leucocyte counts on rabbits at different hours of the same day.

TIME	NO. OF COUNTS	NO. OF RABBITS	MEAN WHITE CELL COUNT	STANDARD ERROR OF MEAN
9 A.M.	30	30	9,367	481
11 A.M.	30	30	10,625	768
1 P.M.	30	30	10,833	881
3 P.M.	22 ¹	22	10,770	888

The white blood cell counts of rabbits as determined by a group of fifteen different investigators were analysed statistically with our data and the results are given in table 3. These data from 1145 counts made upon 466 normal rabbits show that the mean count is 10,228, the standard deviation is 3566 the standard error of the mean is 105 and the coefficient of variation is 34.86%. The mean count as obtained from our data does not differ significantly from the mean count obtained from these composite data.

It is of interest to note that in this sample of 1145 counts it is possible, by logarithmic transformation, to get a good fit with a normal curve. The calculation of the deciles for these data show that 90% of the counts are above 6250 and 90% are below 14,890. Sixty per cent of the counts are between 7389 and 12,794.

TABLE 3
Composite data of leucocyte counts in rabbits.

NAME OF AUTHORS	NUMBER OF RABBITS	NUMBER OF COUNTS	MEAN	STANDARD DEVIATION	STANDARD ERROR OF MEAN	COEF- FICIENT OF VARIATION
						%
Bushnell and Bangs ¹ ('26)	100	100	10,675	2224		20.83
Cunningham et al. ² ('25)	217	53	11,281			
Pearce and Casey ¹ ('30)	1110	174	9,562	2919		30.53
Downs and Eddy ('20)	15	15	10,360	2478	640	23.9
Webb, Williams and Basinger ('10)	18	18	9,800	3129	737	31.9
Wells ('17)	24	24	10,760	4202	859	39.0
Jones ('12)	15	15	6,520	2054	530	31.5
Moss and Brown ('11)	13	135	7,646	2587	222	33.8
Zinsser and Tsen ('17)	24	24	11,120	3403	695	30.6
Brinckerhoff and Tyzzer ('02)	22	22	9,318	2473	527	26.5
Orr ('17)	12	12	8,246	3601	1040	43.6
Ewing ² (1895)	9	9	8,500	1895	631	22.2
Muir ² ('12)	7	7	6,785	3143	1190	46.32
Muir ² ('01)	8	8	7,537	2269	801	30.1
Rogers ² ('20)	13	13	10,366	2173	603	20.9
Bunting ('06)	15	15	9,216	2892	747	31.3
Reifenstein, Ferguson and Weiskotten ('41)	6	42	9,942	2342	361	23.5
Scott and Simon ('24)	100	206	11,505	2198	153	19.1
Haynes; Rigdon	165	580	10,662	3960	164	37.1
Composite data	466	1145	10,228	3566	105	34.86

¹ These data are not included in our composite data since the individual counts are not given in their publications.

² Although standard deviations have been calculated for these samples it should be noted that the samples, by themselves, are too small for any weight to be attached to the constants determined from the samples.

DISCUSSION

The mean number of white blood cells obtained from 580 counts in 165 rabbits is 10,662. This mean is not significantly different from the mean obtained from 1145 counts. These 1145 counts, of course, are made up of the 580 counts from this laboratory and the 565 counts recorded by various investigators in the literature.

It would seem that the term "normal" is usually used too broadly. What is implied, of course, is either "apparently

normal'' or that the animal has not been used recently in any experiment.

Scarborough ('31) compiled the results of eighty-two investigators on the number of white blood cells in rabbits and found the normal to be 7900 cells per cubic millimeter with a normal variation from 4000 to 13,000. In computing these data Scarborough assumed that the number of rabbits used by the different investigators was ten if the number was not stated. Such an assumption, in our opinion, is not warranted. When mean values are given without any information as to the size of the sample they cannot be treated statistically.

The fluctuation during the day in the number of leucocytes of rabbits is indicated by the studies of Reifenstein and his associates ('41). They counted the cells in fourteen rabbits. The number of cells was frequently but not always increased in the afternoon over those of the forenoon. We made a statistical analysis of their counts and found that this increase was not significant. Our counts based upon sixty-two rabbits show a statistically significant increase in the afternoon count over that of the forenoon. Since individual rabbits show such a wide variation in the number of leucocytes a large sample is necessary to show either a significant increase or decrease in the mean count.

Cheng ('30) found that the number of leucocytes in rabbits revealed a tendency toward two daily elevations, one in the forenoon and the second in mid-afternoon. The counts upon which these deductions of Cheng were made are not given in the literature, therefore we cannot study them statistically.

Counts were made upon one group of rabbits in which xylol was applied to the ears. The mean white cell count in these xylol treated rabbits was only slightly more than that in the untreated animals. This difference is not statistically significant on the sample we used. Scott and Simon ('24) used xylol upon the ears of their rabbits. Their mean counts was 11,505. This is the highest mean count in normal rabbits found in the literature (table 3). In view of these data, xylol apparently should not be used on the ears of rabbits included in any ex-

periment where variations in the number of leucocytes are being followed.

Factors influencing the leucocytic count of the rabbit other than the normal fluctuations during the day have been studied. Among these studies are those of Brinckerhoff and Tyzzer ('02) who found a decrease in the number of white cells after 12 hours of fasting. The data when analysed statistically show, however, that this decrease is not significant. Danzer ('30) followed the leucocyte count in twenty-six rabbits during the different seasons of the year and found no significant variation. Cheng ('30) concluded from his data that age influences the number of cells in the rabbit. He observed that rabbits over 6 months of age have a much higher count than rabbits 2 months old or less. Cheng also observed that there was a tendency for rabbits to retain a characteristic leucocyte count throughout life.

SUMMARY

Our data show that the mean leucocyte count for the rabbits examined in this laboratory is 10,662. This figure is based upon 580 counts made upon 165 apparently normal rabbits. Eighty per cent of the counts are between 6530 and 16,148.

When our data are combined with that of fifteen other investigators we have a total of 1145 counts made upon 466 rabbits. The mean leucocyte count from these data is 10,228

There is a significant increase in the number of circulating leucocytes at 1:00 P.M. and 3 P.M. when compared with the count at 9:00 A.M. in samples of sixty-two and fifty-four rabbits.

The fluctuation in the number of white blood cells is large and the variations are marked even when the counts are made at short intervals. These factors always should be considered in the interpretation of any variation in the number of white blood cells in the rabbit.

These data emphasize the importance of large samples if changes in the mean leucocyte count of the rabbit are being studied.

LITERATURE CITED

- BRINCKERHOFF, W. R., AND E. E. TYZZER 1902 On physiological leucocytoses of the rabbit. *J. Med. Research.*, vol. 7, p. 191.
- BUNTING, C. H. 1906 Experimental anaemias in the rabbit. *J. Exper. Med.*, vol. 8, p. 625.
- BUSHNELL, L. D., AND E. F. BANGS 1926 A study in the variation in number of blood cells of normal rabbits. *J. Infect. Dis.*, vol. 39, p. 291.
- CHENG, S. C. 1930 Leucocyte counts in rabbits: Observations on the influence of various physiological factors and pathological conditions. *Am. J. Hyg.*, vol. 11, p. 449.
- CUNNINGHAM, R. S., F. R. SABIN, S. SUGIYAMA AND J. A. KINDWALL 1925 The role of the monocyte in tuberculosis. *Johns Hopkins Hospital Bull.*, vol. 37, p. 231.
- DANZER, M. 1930 Studies on the arneth count. XV. *Quart. J. Exper. Physiol.*, vol. 20, p. 141.
- DOWNES, A. W., AND N. B. EDDY 1920 The influence of splenic extract on the number of corpuscles in the circulating blood. *Am. J. Physiol.*, vol. 51, p. 279.
- EWING, JAMES 1895 Toxic hypoleucocytosis. *New York State J. Med.*, vol. 61, p. 257.
- JONES, C. P. 1911-1912 Observations on the changes produced in the blood and bone marrow by haemorrhage and blood destruction. *J. Path. & Bact.*, vol. 16, p. 48.
- MOSS, W. L., AND G. L. BROWN 1911 Variations in the leucocyte count in normal rabbits, in rabbits following the injection of normal horse serum, and during a cutaneous anaphylactic reaction. *Johns Hopkins Hosp. Bull.*, vol. 22, p. 258.
- MUIR, ROBERT 1901 On the relations of the bone-marrow to leucocyte production and leucocytosis. *J. Path. & Bact.*, vol. 7, p. 161.
- MUIR, ROBERT, AND J. W. M'NEE 1912 The anemia produced by a haemolytic serum. *J. Path. & Bact.*, vol. 16, p. 410.
- ORR, T. G. 1916-1917 Blood picture following experimental splenectomy. *J. Lab. & Clin. Med.*, vol. 2, p. 895.
- PEARCE, LOUISE, AND A. E. CASEY 1930 Studies in the blood cytology of the rabbit. *I. J. Exper. Med.*, vol. 51, p. 83.
- REIFENSTEIN, G. H., J. H. FERGUSON AND H. G. WEISKOTTEN 1941 Studies on leucocytosis. *I. Am. J. Path.*, vol. 17, p. 219.
- ROGERS, J. B. 1920 Experimental studies on the leucocytes with particular reference to tubercle bacillus infections. *Tr. Nat. Tuberc. Ass.*, vol. 16, p. 363.
- SCARBOROUGH, R. A. 1930-1931 The blood picture of normal laboratory animals. *Yale J. Biol. & Med.*, vol. 3, p. 63.
- SCOTT, J. M., AND C. E. SIMON 1924 Experimental measles. *I. Am. J. Hyg.*, vol. 4, p. 559.
- WEBB, G. B., W. W. WILLIAMS AND A. F. BASINGER 1910 Artificial lymphocytosis in tuberculosis. *Tr. Nat. Tuberc. Assoc.*, vol. 6, p. 279.
- WELLS, C. W. 1917 Leucopenia and leucocytosis in rabbits. *J. Infect. Dis.*, vol. 20, p. 219.
- ZINSSER, HANS, AND EDGAR TSEN 1916-1917 On hyperleucocytosis and its bearing on specific therapy. *J. Immunol.*, vol. 2, p. 247.

BOOK REVIEW

THE RAT IN LABORATORY INVESTIGATION. By a staff of thirty contributors, edited by John Q. Griffith, Jr., and Edmond J. Farris. Published by J. B. Lippincott Company, Philadelphia, Montreal, London, 1942. 488 pages. 178 illustrations. Price \$7.50.

This is a relatively small and rather well illustrated book which constitutes a good reference source for those conducting studies involving the rat. The list of thirty contributors includes investigators who have made significant contributions. There are twenty-two chapters covering, among other subjects, breeding, gross anatomy, dietary requirements, teeth, digestive system, technics for the investigation of psychological phenomena, hormones, hematology, circulation, radiology, surgery, histological methods and parasitology.

Certain chapters, for example, those which contain information on breeding methods, anesthesia, handling of animals, running of the laboratory, equipment and surgery of the rat are of general interest. Other chapters, however, are designed primarily for those interested in special fields of investigation, although in no case is the discussion so technical that the average reader cannot benefit from the presentation. There is a rather comprehensive discussion of technics utilized by the psychologist and an excellent review of research on the teeth of the rat. The latter chapter indicates how experimental studies of the teeth may be applied to advantage in fields other than dental research. Dietary requirements and metabolism are simply and rather well summarized. The subject of drug dosage is extensively reviewed with reference to the rat.

There is perhaps an undesirable inequality of emphasis. Approximately 40% of the book is devoted to the teeth, drug dosage and technics used by the psychologist. The rat has proved to be an excellent animal for endocrinological investigations, but relatively little space has been devoted to this subject. Although methods of biological assay are discussed there are no photographs of either gross or microscopic preparations which might serve to illustrate the criteria used in such standardization. In view of the fact that certain portions of the book are very well illustrated the lack of adequate anatomical and histological plates is made more apparent. The subject of histology of the rat is quite neglected, and although the summary on gross

anatomy should be of value from the standpoint of general orientation, it is not of such a nature that it would be of reference value to one interested in the minute surgery or careful dissection of certain regions.

Many subjects omitted might justify as much space as several discussed at great length. No mention is made, for example, of the Horsley-Clarke apparatus, which has been an aid in neurological experiments on the rat, nor of the effects of administration of carcinogenic substances except for one very brief reference.

Extensive bibliographies are appended to several chapters (teeth, pharmacology, nutrition, parasitology, technics for investigating psychological phenomena) and most of the chapters contain important references. There are indications of how the rat can serve as the experimental animal in various fields of investigation in which the rat is not ordinarily used as the test animal. The reader is also made aware of the fact that radiologic and other clinical diagnostic methods can be adapted for use in the study of the rat. The reference value of the book is borne out by the comprehensive summary dealing with the dosage of drugs and the more inclusive chapters such as those on teeth, nutrition, psychological technics and parasitology.

Since the rat has been used in so many fields of research it would be difficult to demonstrate fully in one volume the complete role of this animal in laboratory investigation. Those preparing a book on the rat or any other animal are at a disadvantage since primary interest centers not on the animal but the subjects of investigation. This book does, however, discuss problems and not merely the animal. In general it represents a scholarly contribution and should be of value to the investigator and others associated with the laboratory.

ARTHUR KIRSCHBAUM,
University of Minnesota.

BOOK REVIEW

THE RETINA, S. L. Polyak. 4to. Pp. X and 607, plus 100 plates on 56 leaves. The University of Chicago Press, Chicago, 1941. Price \$10.00.

The scope of this book is indicated in its subtitle: "The Anatomy and the Histology of the Retina in Man, Ape and Monkey, Including the Consideration of Visual Functions, the History of Physiological Optics, and the Histological Laboratory Technique." It represents one phase of an ambitious program that has already run into 9 years of endeavor.

Part I covers the methods by which the retina and visual pathway can be studied. The descriptions are detailed and may appear to some to contain over-much elementary or standard material, to be found in well-known reference works on technique. Yet the text abounds with criticisms, comments and practical suggestions and evaluations. Moreover, the application to the retina and fiber systems is always kept in the forefront, so that the 84 pages are in many ways a unique compilation, based on personal experience. This section will prove useful to others besides those desiring to follow the author into kindred investigative fields.

Part II summarizes in 46 pages the knowledge of the anatomy and physiology of the eye, and of optics, accumulated from classical antiquity to and through the Renaissance. Its preparation has led the author into a major diversion, "which in the opinion of benevolent observers threatened to destroy all hope of ever seeing the accumulated observations organized into a book." Again one must characterize this review as "unique" (the temptation to employ this adjective recurs frequently and justifiedly). Of special importance in tracing the history of the understanding of the eye from the Greeks to the rebirth of investigation in Europe is the careful exposition of the rôle played by the Arabs. The bridge of cultural continuity extended from Byzantium, through the Arabian nations and thence into Spain. During the Dark- and Middle Ages the Arabs not only absorbed the essentials of Greek knowledge and remodeled it to suit their own purposes, but also they diffused this learning so that it eventually made re-entry into Europe. When this occurred, even Vesalius was unable to break loose from the influence of the

heritage of Arabian diagrams of the eye. The rebellion of Vesalius against ancient authority probably is least successful in his account of the eye. In spite of signal improvements, based on personal observations, he perpetuated such errors as a flat cornea, a complete iridial partition and a centrally located lens behind an absurdly huge posterior chamber. These historical researches of Doctor Polyak have brought to light Arabian manuscripts long considered lost, and the accompanying illustrations are now published for the first time.

Part III continues in 43 pages the historical treatment by concentrating on the investigations concerning the structure and function of the retina from the earliest naked-eye observations to the initiation of microscopical study by Leeuwenhoek, and during the succeeding $2\frac{1}{2}$ centuries until the present time. Of paramount importance are the inclusion of critical evaluations of the contemporary state of our knowledge. The fragmentary current information concerning the histoanalysis of the primate retina is reviewed, the speculative rather than evidential nature of present-day diagrams of retinal structure is emphasized and the consequent impossibility of proper functional synthesis, based upon them, is argued in detail.

All of the considerations touched upon in previous paragraphs are but a preamble to the next two sections which deal largely with the author's first-hand contributions to retinal histoanalysis and synthesis. Part IV devotes 153 pages to the minute structure of the retina. Here are presented the findings from the patient and successful studies of Doctor Polyak. They embody the first extended attempt to apply the Golgi and methylene blue techniques to the retina since the time of Cajal and Dogiel. During these decades little or nothing has been added to the analysis of the retina, and our knowledge of retinal synapses remains essentially where it was left by Cajal almost half a century ago. It is probable that the immense prestige of Cajal has served as an effective deterrent to the reopening of inquiry in this difficult field, and too much praise cannot be accorded Doctor Polyak for his courage and tenacity in prosecuting new researches, rather than accepting as final the conclusions of the brilliant Spanish master.

It is impractical to characterize in detail the diverse types of neurones described by Doctor Polyak, and still more so to indicate their intricate synaptic interrelations. Two types of bipolar cells are recognized as polysynaptic, or common rod- and cone varieties, although one of these types has three subvarieties. A second type of bipolar cell is related synaptically to only one cone and is, accordingly, a monosynaptic or individual bipolar. There are one kind of horizontal cell and three kinds of amacrine, although the classification of this latter group is admittedly the least certain of all the cells of the retina. The ganglion cells are either polysynaptic

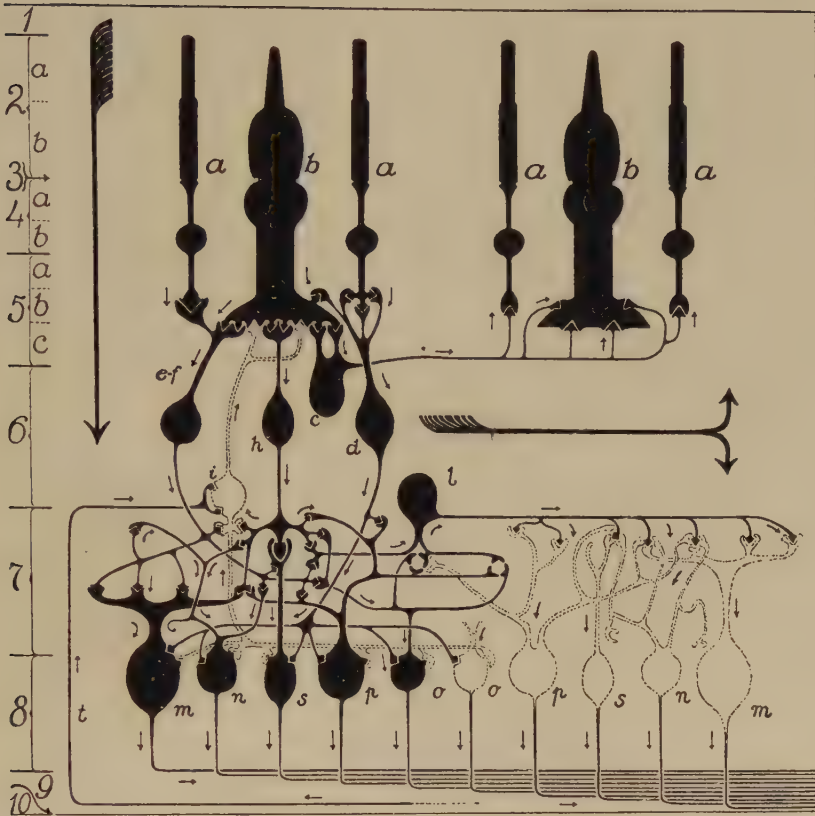


Fig.1 The structure of the primate retina reduced to its essentials, including the synopsis of the propagation of the retinal impulses from the photoreceptors to other parts of the retina, to the brain, and from the brain back to the retina (direction indicated by the arrows). Copied from figure 98 of "The Retina," with the permission of The University of Chicago Press.

Labeling of the cells: (*a*, *b*) rods and cones, or the photoreceptors where the nervous impulses are generated by physical "light" (in the scheme only the left group of the photoreceptors is assumed to be stimulated by light); (*c*) horizontal cells by means of which the impulses are transmitted to the surrounding rods and cones; (*d*, *e*, *f*, *h*) centripetal bipolar cells of the mop, brush, flat, and midget varieties, which "transmit" the impulses from the photoreceptors to the ganglion cells, the bipolars serving as "analyzers"; (*i*) centrifugal bipolar cell, a variety of the "amacrine cells," which probably receives the impulses from the centripetal bipolars, from the ganglion cells, and also from the brain by way of the centrifugal or efferent fibers (*t*) and transmits them back upon the photoreceptors (*a*, *b*); (*l*) an "amacrine cell" which possibly intercepts a part of the bipolar impulses and spreads them over the surrounding territory; (*m*, *n*, *o*, *p*, *s*) ganglion cells which receive impulses from the centripetal bipolars and transmit them to the brain along their axons called "optic nerve fibers."

(diffuse) in nature, with four or five subvarieties, or monosynaptic and in relation with an individual bipolar. Exogenous or centrifugal fibers are not positively identified as such, even though axon-like fibers and telodendron-like terminations were found whose continuity with nerve cells could not be determined. The neuroglia of the retina are analyzed into radial fibers of Müller, incomplete cells of this type ("interstitial spongioblasts"), protoplasmic and fibrous astrocytes, and microglia. It is regrettable in a reference volume of this size and scope that the pigment epithelium is dismissed, together with Bruch's membrane and the choriocapillaris, with four lines!

Just as the retinal cell-types are more numerous than Cajal knew, so also are their interrelations far more intricate than he supposed. The accompanying figure indicates in simplified form the component cells and their synaptic connections. At least thirty-eight kinds of synapses are recognized. The synaptic system is such that the rod cells transmit impulses to all bipolars except the private cone type, and through these to all varieties of ganglion cells. The cone cells discharge impulses to all kinds of bipolars, and through them to all varieties of ganglion cells. In addition, the cones may influence distant rod- and cone cells by way of the horizontal cells. It is held that the synaptic relations suggest that the cone system is organized in a way that is territorially more restricted than is the rod system. Especially in the vicinity of the fovea is each cone linked to a private bipolar that, in turn, is related to a private ganglion cell. This receptor-transmitter apparatus can respond to a localized photic stimulus. The aggregate of such functional units may well supply the structural basis for visual space perception, or visual acuity. On the other hand, the rods, by virtue of their connections, always respond in groups; this is true regardless of the degree of restriction of their stimulation by light. This type of response suggests that the fundamental effect of such group interrelations is the reinforcement of the intensity of excitation — a marked advantage in dim-light vision.

Part V deals with a synthesis of the structure of the retina in relation to visual functions (79 pages). The object of this section, in the words of the author, is:

to interpret the basic visual functions, such as the perception of light, of colors, and of space, in terms of structures of the visual organs, and vice versa. Since this would be an impossible task without a more thorough understanding of the intimate organization of the nervous tissue in general, the latter was assumed as the third principal object in this work.

The aim of the book as outlined, although coinciding in its main lines with the aim of modern neurohistology, neurophysiology, and physiology of vision, has necessitated a much deeper penetration into the problem of histophysiological organization of the nervous gray substance than has been carried out before.

The book endeavors, above all, to find the functional meaning of the morphological variation of the nerve cells and of their combination into complex tissue patterns which, in the aggregate, make up the bulk of the brain and the spinal cord. It was therefore inevitable that, by entering into fields as yet little known, I have arrived, in several respects, at an opinion concerning the fundamental properties of the nervous tissue different from the one generally accepted. I believe, however, that in each case I have stated with sufficient clarity the reasons which have induced me to disagree. Also, I hope that, while so doing, I have shown my intention not to be declaring some immutable dogmas but rather to be opening the roads along which a better understanding of the nervous system in general, and of the visual system in particular, may be achieved.

There are an unusually comprehensive bibliography of 2769 titles and an adequate index. The illustrations, presented as an appendix, comprise 100 plates.

It is difficult to evaluate at this time the true worth of this volume. That it contains the ultimate truth in fact and interpretation, the author has been the first to disclaim. But that it marks a momentous advance in retinal analysis and synthesis cannot be gainsaid. Venturing into the realm of prophecy, one may suggest, without undue temerity, that this work is destined to enter the lists as a strong competitor for future long-term acclaim. Indeed, the possibility even exists that it may eventually be elevated from out the rank of contemporary writings and placed in the select company of anatomical classics.

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